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PROFILING CELL SURFACE SIALYLATION AND DESIALYLATION DYNAMICS OF IMMUNE CELLS

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at the
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ACKNOWLEDGEMENTS

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Sialic acids (SAs) are a diverse family of naturally occurring 2-keto-3-deoxy-nononic acids that are involved in a wide range of biological processes, including early fetal development, cellular recognition, and utilization by microbes. While it is clear that cell surface SAs are highly involved in the immune system, the sialylation status of individual immune cells and functions are still unknown. In this study, I combined the newly developed LC-MS/MS methods with flow cytometry and confocal microscopy to systematically study the sialylation and desialylation dynamics of macrophages at different conditions.

First, I developed an accurate LC-MS/MS method to quantify free SA in human plasma with isotope-labeled standard calibration and 3,4-diaminotoluene derivatization. This method is capable to distinguish SA analogues in complex biological samples, which paves the path for dynamic SAs research. Meanwhile, another LC-MS/MS method with direct SAs quantification was developed for high throughput analysis. This method does not require complicated sample preparation and can quantify SA at 2 ng/mL.

Next, I performed globally profiling of sialylation status of Raw 264.7 macrophages by flow cytometry, confocal microscopy, and LC-MS/MS. Both flow cytometry and confocal microscopy showed the predominant of α-2,3 linked SAs on the cell surface, and
increase of \( \alpha-2,6 \) linked SAs after atorvastatin treatment. Moreover, LC-MS/MS showed total SA increased 3 times upon treatment. Further experiment indicated the correlation of \( \alpha-2,6 \) linked SAs with cell apoptosis.

Finally, I systematically examined the sialylation and desialylation profiles of THP-1 monocytes after differentiation and polarization. Both \( \alpha-2,3 \) and \( \alpha-2,6 \) linked SAs on the cell surface were decreased during differentiation, which was in accordance with the increased free SA in the medium and elevated activity of NEU1 sialidase. Meanwhile, the increase of SA expression during differentiation was evidenced by siaoglycoconjugates inside the cells and total SA in the cell lysate.

Overall, the combined approach has bee successfully applied to profile SAs in the cell culture system. LC-MS/MS can accurately quantify SA in a high throughtput fashion. The SA linkages can be distinguished by flow cytometry and confocal microscopy with specific lectin labelings. The SA levels and linkages provide markers of cells at different status.
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ABBREVIATIONS

4-MU-NANA: 4-Methylumbelliferyl N-acetyl-α-D-neuraminic acid

ACG: Agrocybe cylindracea lectin

AFS: amniotic fluid supernatant

Allo A-II: Allomyrina dichotoma lectin II

Asn: asparagine

Asp: aspartic acid

CMP: cytidine monophosphate

CV: coefficient of variation

DAPI: 4,6-diamidino-2-phenylindole

DAT: 3,4-diaminotoluene

DDB: 1,2-diamino-4,5-dimethoxybenzene

DMB: 1,2-Diamino-4,5-methylenedioxybenzene

DMBA: 4,5-dimethylbenzene-1,2-diamine

DMEM: Dulbecco's Modified Eagle Medium

EBP: elastin-binding protein

EGFR: epidermal growth factor receptor

ER: endoplasmic reticulum

ESI-MS: electrospray ionization-mass spectrometry

FBS: fetal bovine serum

Fuc: fucose

Gal: galactose
β-GAL: β-galactosidase
GalNAc: N-acetylgalactosamine
Glc: glucose
GlcNAc: N-acetylglucosamine
GNE: UDP-GlcNAc 2-epimerase/ManNAc kinase
HMG-CoA: 3-hydroxy-3-methylglutaryl coenzyme A
ILSC: Isotope-labeled standard calibration
IS: internal standard
KDN: 2-keto-3-deoxynononic-acid
LAMP: lysosome associated membrane protein
LC-MS/MS: liquid chromatography coupled mass spectrometry
LFA: Limax flavus lectin
LLOQ: lower limit of quantification
LPS: lipopolysaccharide
MAA: Maackia amurensis lectin
MAH: Maackia amurensis hemagglutinin
MAL: Maackia amurensis leukoagglutinin
MALDI-TOF: matrix assisted laser desorption/ionization-time of flight
Man: mannose
ManNAc: N-Acetylmannosamine
MFI: mean fluorescent intensity
MPA: Macrophomina phaseolina agglutinin
MRM: multiple reaction monitoring
NANP: Neu5Ac-9-P phosphatase
NANS: Neu5Ac-9-P synthase
Neu: neuraminic acid
NEU1: intra lysosomal sialidase
NEU2: cytosolic sialidase
NEU3: plasma membrane-associated sialidase
NEU4: lysosomal or mitochondrial membrane-associated sialidase
Neu5Ac: \(N\)-acetylneuraminic acid
Neu5,9Ac2: \(N\)-acetyl-9-O-acetylneuraminic acid
Neu5Gc: \(N\)-glycolylneuraminic acid
OPD: o-phenylenediamine
PBS: phosphate buffered saline
PDGF: platelet-derived growth factor
PEP: phosphoenolpyruvate
PFA: paraformaldehyde
PI: propidium iodide
PMA: phorbol 12-myristate 13-acetate
PPCA: serine carboxypeptidase protective protein/cathepsin A
PPT: Protein precipitation
PSL: *Polyporus squamosus* lectin
PVL: *Psathyrella velutina* lectin
QC: quality control
RE: relative error
RT: room temperature
SA: Sialic acid
Ser: serine
SNA: *Sambucus nigra* lectin
CHAPTER I
INTRODUCTION TO SIALIC ACID

1.1 Sialic acid

The surfaces of all vertebrate cells in nature comprise a variety of glycoconjugates including glycoproteins, proteoglycans and glycolipids forming the cellular glycocalyx layer, which was original revealed by electron microscopy in 1967 [1, 2]. Sialic acids (SAs) at the “outermost” location of these glycoconjugates are known as the “functional ornament” of the glycocalyx and provide extreme structural and functional diversities (Figure 1). The two major SA core structures are 2-keto-3-deoxynononic-acid (KDN) and neuraminic acid (Neu) sharing nine carbons and differing at the C5-position (Figure 2). The variety of substituents on carbon 4, 5, 7, 8 and 9 generate a diverse family of more than 50 structurally distinct molecules, which can be attached to underlying sugars commonly via α-2,3 or α-2,6 linkage to galactose (Gal), via α-2,6 linkage to N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc), or via α-2,8 linkage to another SA [1, 3-5]. N-acetylneuraminic acid (Neu5Ac) is the most frequent form,
followed by N-glycolylneuraminic acid (Neu5Gc) and O-acetylated derivatives, mostly $N$-acetyl-9-\(O\)-acetylneuraminic acid (Neu5,9Ac2). Among mammals, humans are a known exception in their lack of Neu5Gc, due to an inactivation mutation in a hydroxylase, which modifies CMP-Neu5Ac to CMP-Neu5Gc [6].
Figure 1: The complexity of SA layer on the cell surface. Adapted from reference 4 with permission from Mary Ann Liebert, Inc.
$R_1 = \text{OH in free SA, alpha linkage to Ga} (3/6)$.
$\text{GalNAc (3/6), or SA (8/9)}$

$R_2 = \text{H, or acetyl}$

$R_3 = N$-\text{acetyl (Neu5Ac), N-glycoly (Neu5Gc), hydroxy (KND), or aminc (Neu)}$

$R_4 = \text{H, or acetyl}$

$R_5 = \text{H, acetyl, methyl, sulfate, or SA}$

$R_6 = \text{H, acetyl, lactyl, phosphate, sulfate, or SA}$

Figure 2: Diversity of SA structures and linkages.
Given their remarkable diversities in structures, glycosidic linkages, and underlying glycan chains, as well as their exposed location, it is not surprising that SAs can mediate and modulate a wide variety of physiological and pathological processes, such as immunological process, hormonal response, signal transduction, tumor progression, cell adhesion and protection [7-10]. Firstly, by virtue of the negative charge and hydrophilicity, SAs act as biological masks, which are anti-recognition agents by shielding recognition sites such as sub-terminal galactose residues, antigenic proteins and other macromolecules of cell membranes including receptor molecules. Secondly, SAs function in the opposite way by being biological recognition sites for a great variety of proteins, which are collectively called lectins. Angata et al. proposed to group these glycan recognition proteins into three categories: vertebrate pathogen lectins, vertebrate endogenous lectins, and lectins from other sources such as plants, protostomes, etc. [3] Vertebrate pathogen lectins generally show more specificity on SA structures compared to vertebrate endogenous lectins. For example, most influenza viruses A that spread among birds preferentially recognize SA with α-2,3 linkage to underlying sugar. Humans are resistant to infection at least partly by such virus because we instead display α-2,6 linked SA on the epithelium of upper airways [11, 12]. Another example is the lectins, named adhesions in bacteria, expressed by E. coli K99 strain shows high specificity towards Neu5Gcα2,3Galβ1,4Glc structure on glycolipids, which is abundantly expressed in the gastrointestinal tract of piglets [13]. Plant lectins are traditionally powerful tool to study glycan structures. Their high specificity is able to distinguish isomeric glycans with identical sugar compositions. For example, Sambucus nigra agglutinin (SNA) binds in a highly selective fashion to SA linked to either Gal or GalNAc via α-2,6 linkage. In contrast
to SNA, the family of lectins from *Maackia amurensis* seeds (MAA) recognize α2,3-linked SA [14].

Vertebrate endogenous lectins are usually found in immune system. The most studied lectins include factor H, selectins, and siglecs. Factor H is one of the first discovered vertebrate lectins. Factor H is a “self” identification marker that binds to cell surface SAs to protect cells from the alternative complement pathway [15, 16]. Selectins, a family of three type-I cell surface glycoproteins including E-, L- and P-selectin, participate in many cell-cell interactions in immunity, haemostasis and inflammation [17]. Leukocyte (L)-selectin, which is constitutively expressed on many types of leukocytes, can recognize the sialylated ligands on endothelial venules and then facilitate homing of the cells into lymph nodes. Moreover, independent evidence shows leukocyte adhesion to activated endothelial cells is also mediated by endothelial (E)-selectin which recognize SAs on the leukocytes [18]. However, E-selectin is not constitutively expressed, and requires transcriptional induction by various inflammatory stimuli. Platelet (P)-selectin, which is stored in α-granules of platelets and Weibel-Palade bodies of endothelial cells, can rapidly mobilize to the cell surface in response to various stimuli, again mediating adhesion to SA-containing ligands on leukocytes [17]. Siglecs are a family of SA-binding immunoglobulin-like receptors that participate in the discrimination of “self” and “non-self” and regulate the functions of cells in the innate and adaptive immune systems through recognition of their glycan ligands [19-21]. To date 14 siglecs have been identified in humans, and show highly restricted expression in immune system and the differential expression in various immune cells, such as sialoadhesin in macrophages, CD22 in B cells, MAG in oligodendrocytes and schwann cells, CD33 in macrophages and granulocytes, *etc.*
Sialoadhesion, CD22, MAG and Siglec-15 are conserved across mammals, while the group of CD33-related Siglecs show a great diversity among the species. Most Siglecs are typically bound to sialylated glycans on the same cell surface (*cis* interaction). However, another cell surface or a soluble ligand with a high enough density of sialylated ligands can compete out the *cis* ligand and cause engagement [19]. This can provide insight why SAs are found in so many bacterial and viruses, such as group B *streptococcus*, *Neisseria meningitides*, HIV, porcine reproductive and respiratory syndrome virus, etc. [20-23] On one hand, SAs could provide the pathogen with suppression of the alternative pathway via Factor H, hijack host Siglecs, and get into the host cells. On the other hand, pathogens also use host sialylated structures as ligands for binding and recognition. For example, influenza virus binds to hosts SAs via hemagglutinin, and then cleave the SAs on the same receptors with sialidase to achieve successful infection [24].
Figure 3: Multifarious roles of SAs in immunity. (A) Structure of Neu5Ac, the most common SA in mammals. (B) The high density of SAs on cell surface imparts negative charge and hydrophilicity to cells. (C) Factor H binds cell surface SAs, protecting cells from the alternative complement pathway. (D) Selectins on endothelia and leukocytes initiate leukocytes rolling on endothelial surfaces. (E) Siglecs on immune cells detect sialylated ligands and can inhibit immune cell activation. (F) Host SAs are frequently exploited as attachment sites for pathogens. (G) Microbial sialidases can help pathogens to expose underlying glycan-binding sites, to avoid sialylated decoys. (H) Endogenous sialidases can modulate immune cell functions by modulating receptor clustering, possibly...
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1.2 Sialylation

In general, SAs are synthesized in the cytosol and then presented at the terminal of glycoproteins and glycolipids anchored on the cell surface (Figure 4) [25]. The biosynthesis of SAs starts with UDP-GlcNAc, which is the key compound of the whole amino sugar metabolism. This nucleotide sugar is formed by the hexosamine pathway, which splits off from glycolysis at the stage of fructose 6-phosphate. A minor portion of UDP-GlcNAc participates into SAs synthesis pathway. The formation of Neu5Ac from UDP-GlcNAc is carried out in the cytosol, and requires three enzymes with four enzymatic steps. N-Acetylmannosamine (ManNAc) is a dedicated metabolic precursor for the Neu5Ac synthesis with no other roles in cells. The first two steps of the enzymatic reactions are catalyzed by a bifunctional enzyme called UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE) with both hydrolyzing UDP-GlcNAc 2-epimerase and ManNAc kinase activities. The epimerase function of the GNE converts UDP-GlcNAc to ManNAc with removal of the UDP moiety and epimerization of the GlcNAc. The kinase function of GNE then phosphorylates ManNAc to form ManNAc-6-P. The Neu5Ac-9-P synthase (NANS) uses ManNAc-6-P to catalyzes an aldol addition with phosphoenolpyruvate (PEP) for the formation of Neu5Ac-9-P. This enzymatic reaction is followed by the release of the 9-phosphate group from Neu5Ac-9-P by Neu5Ac-9-P phosphatase (NANP). The pathway then moves from cytosol to nucleus where the de novo synthesized Neu5Ac is converted into CMP-Neu5Ac by the CMP-Neu5Ac synthetase. Lastly, the nucleotide sugar is transported to the Golgi apparatus by the CMP-SA transporter and is served as substrate for different sialyltransferases to install α-2,3, α-2,6, or α-2,8 linked sialosides into glycoproteins or glycolipids [26-28]. However, the biosynthesis of sialyl glycoconjugates
is complicated by the additional modifications on SA either before or after the formation of sialyl linkages in the Golgi, such as the formation of non-human Neu5Gc in animals and O-acetylation in different hydroxyl groups [29-30].

Sialyltransferases are enzymes that directly control the cell surface sialylation patterns, which are typically insensitive to changes in metabolic flux because the Golgi concentration of CMP-Neu5Ac exceeds the \( Km \) for sialyltransferases [31]. Consequently, sialyltransferases are the targets that cells use to regulate their sialylation status in response to variable physiological and pathological stimuli. Twenty distinct sialyltransferases have been identified in both human and murine genomes, and all of them are type II transmembrane glycoproteins that predominantly reside in the trans-Golgi compartment [32]. Based on the type of linkage formation and nature of sugar acceptor, these enzymes can be categorized into four distinct families: ST3Gal family, ST6Gal family, ST6GalNAc family, and ST8SA family [27, 33-35]. All the described ST3Gal family transfer Neu5Ac residues in \( \alpha-2,3 \) linkage to terminal Gal residues found in glycoproteins and glycolipids. In this family, the ST3Gal I and II subfamilies use exclusively the type 3 oligosaccharide structure Gal\( \beta1,3 \)GalNAc-R, whereas the ST3Gal III, IV, V, and VI use the oligosaccharide isomers Gal\( \beta1,3/4 \)GlcNAc-R. The enzymes of ST6Gal family comprise only two subfamilies, ST6Gal I and II, which both use the Gal\( \beta1,4 \)GlcNAc-R as the acceptor substrate. ST6GalNAc family catalyze the transfer of Neu5Ac residues in \( \alpha-2,6 \) linkage to the GalNAc residues found in \( O \)-glycosylproteins (ST6GalNAc I, II, and IV) and glycolipids (ST6GalNAc III, V, and VI). The six members of ST8SA family mediate the transfer of Neu5Ac residues in \( \alpha-2,8 \) linkage to other Neu5Ac residues found in
glycoproteins and glycolipids, among which ST8Sia II and ST8Sia IV are polysialyltransferases.
Figure 4: Overview of mammalian SAs metabolism. Adapted from reference 25 with permission from Oxford University Press.
1.3 Desialylation

Sialidases are a family of exo-glycosidases that catalyze the hydrolytic cleavage of non-reducing SA residues linked to the glycan structures of glycoproteins, glycolipids, and oligosaccharides. They are widely distributed in nature, from virus and microorganisms to vertebrates, but absent in plants, insects, and yeast [36, 37]. Four types of mammalian sialidases have been identified and characterized to date, and the classification is based on their subcellular localization, namely the intra lysosomal sialidase (NEU1), the cytosolic sialidase (NEU2), the plasma membrane-associated sialidase (NEU3), and the lysosomal or mitochondrial membrane-associated sialidase (NEU4) (Table I) [38]. NEU1 is ubiquitously but differentially expressed in tissues and cells, and has been purified from many sources, such as placenta, mammary gland, brain, and liver [38, 39]. In human tissues, NEU1 generally shows strongest expression, 10-20 times higher than those of NEU3 and NEU4, whereas NEU2 expression is extremely low and only reported in a range of tissues such as placenta and testis [40, 41]. NEU3 shows the highest expression in adrenal gland, skeletal muscle, heart, testis, and thymus [42]. NEU4 has the highest expression in brain, skeletal muscle, heart, placenta, and liver [43].
TABLE I: General properties of four mammalian sialidases.

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<td>Cytosol</td>
<td>Plasma membranes</td>
<td>Lysosomes, Mitochondria and ER</td>
</tr>
<tr>
<td>Good substrates</td>
<td>Oligosaccharides, Glycopeptides</td>
<td>Oligosaccharides, Glycoproteins, Gangliosides</td>
<td>Gangliosides</td>
<td>Oligosaccharides, Glycoproteins, Gangliosides</td>
</tr>
<tr>
<td>Optimal pH</td>
<td>4.4-4.6</td>
<td>6.0-6.5</td>
<td>4.5-4.7</td>
<td>4.5-4.7</td>
</tr>
<tr>
<td>Proposed major functions</td>
<td>Degradation in lysosomes; Exocytosis; Immune function; Elastic fiber assembly; Carcinogenesis</td>
<td>Myoblast differentiation</td>
<td>Neuronal and muscle differentiation; Apoptosis;</td>
<td>Neuronal differentiation; Apoptosis</td>
</tr>
</tbody>
</table>
1.3.1 NEU1

Studies carried out so far have demonstrated the distinct target substrates and biological functions among all these four mammalian sialidases [44]. NEU1 is the only sialidase that functions in a multienzyme complex containing at least two other hydrolases including the glycosidase β-galactosidase (β-GAL) and the serine carboxypeptidase protective protein/cathepsin A (PPCA). In acidic condition, such as lysosomal environment, PPCA can promote the formation of NEU1 oligomers, which are catalytically active against sialylated oligosaccharides and glycoproteins with a preference for α-2,3 and α-2,6 sialyl linkages [45, 46]. The role of β-GAL in the multienzyme complex is not so clear, but it may provide a means of regulation of their activities, or may enhance their stability. NEU1 is crucial for the lysosomal catabolism of sialylated glycoconjugates. The defective or deficient enzyme activity is associated with two neurodegenerative lysosomal storage disorders, sialidosis and galactosialidosis (GS). Sialidosis is caused by structural lesions in the NEU1 gene, while GS is a combined deficiency of NEU1 and β-GAL caused by the absence of functional PPCA [47, 48]. Except the well-studied lysosomal catabolic function, the emerging studies mainly based on sialidase deficient animal models have revealed new roles of NEU1 in diverse cellular regulatory pathways including exocytosis, immune function, elastic fiber assembly, and carcinogenesis.

1.3.1.1 Exocytosis

NEU1 negatively regulates lysosomal exocytosis, a cellular process for the recruitment of lysosome to the plasma membrane, resulting in an increase in extracellular proteolytic activity [49, 50]. Neu1 (-/-) mice showed enhanced secretion of lysosomal
proteases and glycosidases and an increased presence of heavily sialylated lysosome associated membrane protein 1 (LAMP-1), which was identified as a target substrate of NEU1. In contrast, silencing of LAMP-1 expression in the cells leads to normalization of the LAMP-1 distribution and reduction in lysosomal exocytosis [49]. Furthermore, the increased lysosomal exocytosis in Neu1 (-/-) mice was linked to reduced endolymphatic potential, dysfunction of transduction in sensory hair cells and hearing loss that were also observed in sialidosis patients [51]. Interestingly, a recent published study showed sarcoma cells gained invasive and drug-resistant abilities by inducing lysosomal exocytosis [52].

Down-regulation of NEU1 and accumulation of oversialylated LAMP-1, tumor cells exacerbate lysosomal exocytosis of soluble hydrolases and exosomes. This facilitates matrix invasion and propagation of invasive signals, and purging of lysosomotropic chemotherapeutics. These malignant traits were reversed by inhibiting lysosomal exocytosis in Neu1 haploinsufficient mice.

### 1.3.1.2 Immune function

Cell surface SAs are implicated as potential modulator of immune cell interaction. It has been known for decades that sialidase treatment could substantially enhance the capacity of resting B cells to stimulate the proliferation of allogeneic and antigen specific, syngeneic T cells [53, 54]. It is suggested that sialidase treatment may be an analogue for an authentic step in B cell activation, which shows specific loss of α-2,6 linked SA in highly activated B cells. Moreover, different studies have shown the NEU1 on the surface of activated T cells is essential to general cell surface desialylation and to production of cytokines such as interleukin 4 (IL-4) and interferon γ (IFN-γ) [55, 56]. Further, the
presenting of NEU1 is required for T cells interaction with antigen presenting cells and converting the group specific component protein into a factor necessary for the inflammation-primed activation of macrophages [57, 58].

Our study and several other studies have demonstrated during the differentiation of circulating blood monocytes and monocytic cell lines into macrophages, the NEU1 activity is dramatically increased, while NEU2, NEU3, and NEU4 are unchanged or even reduced [59-61]. In this process, NEU1 and its activator PPCA were first targeted to the lysosome and then sorted to the LAMP-2 negative and MHC II positive vesicles, which later merged with the plasma membrane [60]. In line with this finding, the genetic approach showed macrophages and immature dendritic cells from NEU1-deficient mice presenting increased sialylation of the cell surface and compromised ability to phagocytosis [62]. As expected, this phenotype was reversed by the exogenous NEU1 treatment, which can affect the transduction of signals from the Fc receptors for immunoglobulin G via desialylation. Another NEU1 regulated immune receptor is Toll-like receptors (TLR) that play key roles in activating immune response during infection [63, 64]. Ligand binding to TLR-2, -3, and -4 rapidly induces NEU1 activity that promotes intracellular signaling [63, 64]. This activity is speculated due to the removing of SA from TLR by NEU1. Later study reveals a broad repression of TLR function by Siglecs and a sialidase-mediated de-repression that allows positive feedback of TLR activation during infection [65].

1.3.1.3 Elastic fiber assemble

The elastin-binding protein (EBP), identical to a spliced variant of β-GAL, forms a cell surface elastin receptor with PPCA and NEU1. During the formation of elastic fibers,
the EBP initially plays the role of intracellular chaperone for hydrophobic and non-
glycosylated tropoelastin, protecting it from premature self-aggregation and degradation
by elastases, and facilitating its extracellular deposition on the microfibrillar scaffold [66,
67]. The cell surface-residing NEU1, which is presumably activated by the binding of EBP
tropoelastin complex or free SA generated from undetermined substrates [68], catalyzes
the removal of the terminal SA from carbohydrate chains of microfibrillar protein, forming
the structural scaffold of new elastic fiber, unmasking their penultimate galactose. The
exposed Gal then interacts with the galactolectin domain of the EBP, thereby inducing the
release of transported tropoelastin molecules and facilitating their subsequent assembly
into elastic fiber. Further studies with NEU1 deficient fibroblasts from sialidosis and GS
patients showed significantly less insoluble elastin as compared to control fibroblasts, but
had increased levels of soluble tropoelastin [66, 69]. This phenotype was reversed by the
addition of exogenous sialidase in the medium.

1.3.1.4 Carcinogenesis

Altered sialylation of tumor cell surface glycoconjugates has been described to be
highly associated with the metastatic phenotype of cancers. In particularly, decreasing
NEU1 expression is one of the characteristic features for multiple cancers, while with an
inverse correlation with metastatic ability [44, 70-72]. The level of NEU1 activity and
expression in different clones of transformed rat fibroblast 3Y1 cells and mouse
adenocarcinoma colon CT26 cells inversely correlated with their metastatic potential [73,
74]. Those transformed with v-fos had lower sialidase activity and higher metastatic
potential [73]. Further study from the same group demonstrated that overexpression of
NEU1 in mouse B16 melanoma cells reversed their metastatic capacity as detected by the suppression of the pulmonary metastasis in mice, invasiveness in collagen gels, and motility on colloidal gold-coated glass plates [75]. Another work showed that NEU1 overexpression in colon cancer HT-29 cells significantly reduced their liver metastasis potential in mice as well as migration, invasion, and adhesion properties in vitro, whereas NEU1 silencing caused the opposite effect [76]. One of the target molecules of NEU1 has been identified to be laminin receptor, integrin β4, which undergoes desialylation and decreased phosphorylation followed by attenuation of the focal adhesion kinases and extracellular signal regulated kinase 1/2 (ERK 1/2) pathway, and downregulation of matrix metalloproteinase-7 [76]. Interestingly, a microRNA, miR-125b, targeting eight transcripts including NEU1 as metastatic suppressor, was found to promote growth of prostate cancers in both intact and castrated male nude mice by down-regulating pro-apoptotic and tumor suppressor genes [77]. Although many experiments do not identify the molecular mechanism of the observed changes in metastatic capacity, they implicate NEU1 as a negative regulator of malignant properties of cancer cells.

However, NEU1 does not always act as a cancer suppressor. A recent study found that NEU1 was expressed in a higher level in ovarian cancer tissues than that in adjacent normal tissues [78]. The inhibition of NEU1 with siRNA can effectively prevent the proliferation, apoptosis, and invasion of human ovarian cancer cells, which was speculated by targeting lysosome and oxidative phosphorylation signaling pathway [78]. Moreover, in another proposed signaling paradigm, NEU1 was activated by an epidermal growth factor receptor (EGFR)-induced G protein coupled receptor signaling process and metalloproteinase-9 activation. This tripartite complex of G protein coupled receptor,
MMP-9, and NEU1 forms an alliance with EGFR tethered at the ectodomain of the receptor on the cell surface. Active NEU1 in the complex with EGFR hydrolyzes α-2,3 SA residues on the receptors, enabling removal of steric hindrance of receptor association and allowing subsequent dimerization, activation, and cellular signaling [79-81]. Subsequently, therapeutic inhibiting of NEU1 with oseltamivir phosphate at the EGFR level disabled the intrinsic signaling platform for cancer cell survival in human pancreatic cancer with acquired chemoresistance [80, 81].

1.3.2 NEU2

NEU2 is the first example of a mammalian sialidase that cDNA cloning is achieved based on the peptide sequence of the protein purified from rat skeletal muscle, where it shows predominant expression [82]. Overexpression of human NEU2 homologue in E. coli and the purification of the enzyme to homogeneity permit its detailed kinetic characterization [83]. The highest catalytic activity and affinity are exhibited on α-2,3 linkage presenting in different sialoconjugates (gangliosides, glycoproteins, sialyl-lactose), whereas the α-2,8 linkage in colominic acid and the α-2,6 linkage carried by α-2,6 sialyllactose are resistant to hydrolysis. The enzyme exhibits the highest activity on gangliosides (GM3, GD1a, GD1b, GT1b, and α2, 3-sialylparagloboside) in their micellar (vesicular in the case of GM3) supramolecular organization. Remarkably, GM1 and GM2 are recognized only as monomers with $K_m$ values as low as 10 nM, while micellar-form monosialoganglioside GM2 and GM1 are not affected by NEU2.

Because of its almost exclusive expression in musculoskeletal tissues, the main stream of NEU2 study is focused on the involvement in myoblast differentiation. The
initial in vitro evidence was obtained from rat L6 myogenic cells [84]. In this regard, an increased NEU2 gene transcription in L6 myoblasts appeared to be dependent on the presence in the promoter region of two pairs of E-box sequence, which were known binding sites for muscle-specific transcription factors involved in differentiation. Subsequently, a similar transcriptional NEU2 upregulation was observed during differentiation of murine C2C12 myoblasts [85]. NEU2 overexpressing clones showed decrement of cell proliferation while enhanced spontaneous myoblast differentiation, which was evidenced by the formation of myogenin-positive myotubes and a significant decrease in the nuclear levels of cyclin D1 protein. The possible involvement of NEU2 in muscle regeneration in vivo has been suggested in the dysferlin-deficient mouse model [86]. In this mouse model for human dysferlinopathy the down-regulation of the NEU2 gene was associated with impairment of muscle regeneration. These results provide evidences that NEU2 upregulation constitutes an important event to trigger in vitro myoblast differentiation. In addition, NEU2 has been suggested to drive insulin-like growth factor 1-induced hypertrophy of myoblasts [87]. In this signal pathway, the activation of the phosphatidylinositol-3-kinase-serine-threonine protein kinase AKT1/mammalian target of rapamycin pathway leaded to an increase of NEU2 level. This upregulation could be obtained by using a constitutively activated form of AKT1, whereas transfection of a kinase-inactive mutant of AKT1 inhibited myotube formation and causes NEU2 down-regulation. Furthermore, the enzymatic activity of NEU2 is inversely regulated during in vitro myoblast hypertrophy and atrophy [88]. As expected, hypertrophy was found to be coupled to high levels of NEU2, whereas atrophy was characterized by a down-regulation of the enzyme. However, it is still uncertain how NEU2 actually functions in human tissues.
and cells due to the extremely low expression. The biological and phylogenic significance of marked decrease in NEU2 expression in the human tissues thus remains to be elucidated.

1.3.3 NEU3

NEU3 is membrane-associated sialidase that ubiquitously expressed in various of animal species [38, 42]. It possesses specific activity for gangliosides, including GD3, GD1a, GD1b and GM3; whereas sialyllactose, 4MU-NeuAc, GM1 and GM2 are poor substrates, and it has no activity against sialylated glycoproteins such as fetuin, transferrin and orosomucoid [89, 90]. Interestingly, the mouse NEU3, but not NUE2, seems to remove even stable SA of GM1 and GM2 in the presence of GM2 activator protein [91]. Moreover, the activity of sialidase NEU3 is exerted also on gangliosides exposed on the extracellular leaflet of the plasma membrane of adjacent cells by cell-cell interaction [92]. The puzzling mechanism of NEU3 anchorage to the membrane was solved by studying the overexpressed mouse NEU3 in HeLa and COS-7 cells [93]. NEU3 behaves as a peripherally associated membrane protein, presenting in both the plasma membrane and the membranous structures corresponding to the recycling endosomal compartment, from which it can be released by treatment with carbonate.

1.3.3.1 Regulation in membrane microdomains

NEU3 has been suggested to play essential roles in regulation of cell surface functions because of its major localization in the plasma membrane and strict substrate preference for gangliosides involved in signal transduction. NEU3 is located in rafts of neuroblastoma cells and in caveolae of HeLa cells, closely associated with caveolin-1,
probably through the caveolin-binding region [94, 95]. In caveolae, NEU3 has been proposed to control platelet-derived growth factor (PDGF)-induced Src mitogenic signaling and DNA synthesis by modification of GM1 levels at cell surfaces [96]. Caveolin-enriched membrane microdomains regulate the association of Src family protein tyrosine kinases with PDGF receptor, which represents a pivotal site for kinase activation and cell cycle progression. A member of the transmembrane adaptor protein family, phosphoprotein associated with glycosphingolipid-enriched microdomains, induces NEU3 sialidase activity, leading to GM1 accumulations and PDGF receptor exclusion from caveolae. The biological action of NEU3 is exerted through its activity on gangliosides, which are essential structural and functional components of membrane microdomains. Moreover, NEU3 is also found in tetraspanin-enriched microdomains (TERMs) [96]. Tetraspanins are a family of membrane proteins involved in membrane compartmentalization dynamics. Overexpression of NEU3 in HB2 mammary epithelial cells affected the stability of CD82-containing TERMS, which showed the decreased association with CD151, but enhanced association with EGFR. These results have demonstrated the relevance of gangliosides, as NEU3 substrates, in maintaining the integrity and functionality of CD82-enriched microdomains and suggested a role of NEU3 as possible physiological regulator of these membrane structures.

1.3.3.2 Promotion cell differentiation

The possibly important role of NEU3 in the proliferation control and differentiation in neuronal cell system was revealed several years before the cloning of its cDNA [97]. The direct participation of NEU3 in neuronal cell differentiation was further proved by the
stable transfection of the sialidase in Neuro2a cells, which showed the accelerating neurite arborization following 5-bromodeoxyuridine treatment [98]. Recent studies provide important details on the role played by NEU3 in axonal growth and regeneration via regulation of GM1 level [99, 100]. GM1 has been shown to promote axon growth and regeneration in vitro and in vivo. In contrast, the polysialylated gangliosides, GD1a and GT1b, have been shown to act as receptors for myelin-associated inhibitors [101]. Application of exogenous sialidase that cleaves SA residues from polysialylated gangliosides to generate monosialylated GM1 has been shown to relieve myelin-dependent neuronal outgrowth inhibition as well as promote axon regeneration and functional recovery in rats that have been subjected to spinal cord injury [100, 102]. Moreover, NEU3 asymmetrically accumulating at the tip of one neurite of the unpolarized rat neuron can induce actin instability, thus triggers the polarization of neurons [103]. Suppressing the enzymatic activity blocks axonal generation, whereas stimulating it accelerates the formation of a single (not several) axon [103]. Underlying molecular mechanism indicates NEU3 induces axon specification by enhancing TrkA activity locally, which triggers phosphatidylinositol-3-kinase- and Rac1-dependent inhibition of RhoA signaling and the consequent actin depolymerization in one neurite only.

NEU3 also plays a key role in skeletal muscle differentiation by strictly modulating the ganglioside content, with special regard to GM3 [104-106]. Induced down-regulation of NEU3 in murine skeletal muscle cells C2C12 myoblasts, even when partial, totally inhibits their capability to differentiate by increasing the GM3 level above a critical point, consequently leading to EGFR inhibition and ultimately down-regulation, and a higher responsiveness of myoblasts to the apoptotic stimuli [104]. Conversely, the over expression
of NEU3 affects the EGF signaling pathway in an opposite direction, which promoting cell proliferation and delaying the beginning of the differentiation [105-106]. Regarding this point, NEU3 is proposed as a cancer marker, because of its up-regulation has been found to promote the suppression of cell apoptosis in human cancers by a mechanism dependent on the depletion of gangliosides and subsequent over-activation of mitogenic receptor [71, 107-109].

1.3.4 NEU4

Human NEU4 is present in two isoforms, long (NEU4L) and short (NEU4S), which are different in the presence or absence of 12 amino acids at the N-terminal, and the subcellular location with NEU4L showing a mitochondrial localization and NEU4S in association with endoplasmic reticulum (ER) [110, 111]. The isoforms are differentially expressed in a tissue-specific manner. The brain, muscle and kidney contain both, whereas the liver and colon possess predominantly the short form [110]. Although both isoforms possess broad substrate specificity from glycoproteins to gangliosides and oligosaccharides, they show reduced activity towards substrates containing α-2,6 linked SA and branched Fuc residues [43, 112]. Interestingly, the mitochondrial localization of NEU4L suggests that the protein could be involved in the pathways of mitochondrial apoptosis through the regulation of the level of apoptogenic ganglioside GD3 within the organelle [113, 114]. Mouse NEU4 also has two isoforms derived from alternative splicing, designated NEU4a and NEU4b. However, the mouse Neu4 gene is expressed dominantly in the brain, and only very low levels in other tissues [115]. The expression level of mouse
gene increases 3-14 days after birth suggesting a role for brain development, presumably through digestion of polysialic acids on surfaces of neuronal cell [116].

In addition to the mitochondria and ER membrane localization, NEU4 has also been found in the lysosome lumen. In contrast to NEU1, NEU4 is targeted to lysosomes by the Man 6-phosphate receptor and does not require association with other proteins for enzymatic activity. Expression of NEU4 in the cells of sialidosis and galactosialidosis patients resulted in clearance of storage materials from lysosomes, and this rescue activity exerted by NEU4 was further supported by results obtained from Neu4 deficient cells [43, 117]. In Neu4 knockout mice model, a marked vacuolization and lysosomal storage in lung and spleen cells were observed [118]. In addition, gangliosides profile in the brain showed an increased level of GD1a and a decrease of GM1, suggesting a possible involvement of the enzyme in the ganglioside desialylation processes within the central nervous system. Unlike human Tay-Sachs disease, β-hexosaminidase A-deficient mice remain asymptomatic to 1 year of age, because the catabolism of GM2 can be partially compensated by NEU4 pathway. As expected, mice with a double deficiency of NEU4 and β-hexosaminidase A suffered epileptic seizures and rapid neuronal loss accompanied with GM2 accumulation [118].

The mitochondria localization and predominantly brain distribution of NEU4L suggest it may have important functions in the brain. In contrast to NEU3, murine NEU4 appears to negatively regulate neurite formation, being down-regulated in Neuro2a cells during retinoic acid induced differentiation [116]. Furthermore, overexpression of NEU4 resulted in suppression of neurite formation, and its knockdown showed the acceleration [116]. Interestingly, in this process the endogenous substrate for NEU4 was not
gangliosides but a 95-kDa glycoprotein, which decreased with cell differentiation. A more recent study has shown NEU4 regulated neuronal differentiation is at least partially through degradation of polysialic acids [119]. This mechanism was evidenced in neuroblastoma Neuro2a cells that were co-transfected with Neu4 and a polysialyltransferases, ST8SiaIV. In mouse embryonic hippocampal primary neurons, the endogenously expressed NEU4 was found to decrease during the neuronal differentiation. Moreover, NEU4 was partially co-localized with polysialic acid in neurites and significantly suppressed their outgrowth, whereas silencing of Neu4 showed the acceleration together with an increase in polysialic acid expression.


1.4 Analytical approach

Recent studies are unexpectedly revealing that sialoglycoconjugates contribute in both general and specific ways to many biological regulatory pathways. This dramatic development demands more specific, accuracy, and systematic SA detection technologies. As is described before, both sialyltransferases and sialidases can reflect the total expression level of SA, and may reveal the underlying molecular mechanisms as well. Other more desirable approaches should be able to distinguish SA in different locations (such as cell surface, cytoplasm, and culture medium), different linkages (such as α-2,3 and α-2,6), and even different underlying structures. First of all, SA on the cell surfaces can be monitored by several methods, including lectin labeling, boronic acid capturing, metabolic engineering, and chemical modification [120]. Lectins specifically recognize non-reducing ends of naturally occurring glycans of glycoconjugates, including glycoproteins and glycopeptides. However, they can be very large proteins that may suffer from relatively low affinities, and many lectins can perturb cells by cross-linking receptors, which limits their application in live cells [121, 122]. Nevertheless, these drawbacks can be compensated by another carbohydrate recognition molecule, boronic acid, which forms cyclic esters with diols in different positions of sugars in aqueous solution [123]. Phenylboronic acid selectively binds to SAs and thus has been considered as an innovating molecular targeting platform for SA detection [124]. Metabolic labeling with SA or SA precursor analogues allows the introduction of unique functional groups that provide specific and sensitive detection. However, the poor incorporation efficiency rises great challenge for the sugar structures as well as the application in broad range of cells [125]. Mass spectrometry is another powerful tool that can quantify SA either with or without
chemical derivatization, and differentiate SA in various underlying glycan structures [126]. Undoubtedly, mass spectrometry is the potential answer to more complicated questions arised in SA research.

1.4.1 Lectin-affinity approach to determine SAs on cell surface

SA-specific plant lectins possess historical importance in investigating the expression and biology of SA [121]. During the past decades, lectins that discriminate various types of sialylated glycans have been reported (Table II). Since α-2,3 and α-2,6 are two major forms of SA linkages principally to Gal or GalNAc residues, the lectin searching has been mostly focus on these two types of sialylated glycoconjugates. Wheat germ agglutinin (WGA) is initially identified having high specificity towards GlcNAc and its β-1,4 linked oligosaccharides. Due to configuration similarities to GlcNAc, SA also shows direct interaction with WGA regardless the underling linkages [127, 128]. The lectin from Sambucus nigra (SNA) shows a marked preference for the Neu5Acα2,6Gal/GalNAc over the α-2,3 structure, thus it has been wildly used to differentiate SA isomers on cell surface [129]. The Polyporus squamosus lectin (PSL) has specificity for Neu5Acα2,6Gal over the Neu5Acα2,3 sequence, but unlike SNA, it does not bind to the mucin-derived O-linked Neu5Acα2,6GalNAc sequence. The strict specificity of the lectin for α-2,6 linked SA renders this lectin a valuable tool for glycobiological studies and cancer research [130, 131]. Allomyrina dichotoma lectin II (Allo A-II) is another lectin with high specificity for α-2,6 structure, although it shows a small degree interaction with Galβ1,4GlcNAc [132]. It provides a useful tool to separate complex type oligosaccharides with the
Neu5Acα2,6Galβ1,4GlcNAc group from their isomeric oligosaccharides with the Neu5Acα2,3Galβ1,4GlcNAc group.

Lectins from *Maackia amurensis* provide powerful tool for α-2,3 linked SA structure study. *Maackia amurensis* lectins (MAA) consists of two molecular species, a strongly hemagglutinating hemagglutinin (MAH) and a strongly mitogenic hemagglutinin (MAL). MAH binds preferentially to sialylated O-linked glycans containing the trisaccharide Neu5Aca2,3Galβ1,3GalNAc; MAL preferentially bind to sialylated N-linked or core 2 O-linked glycans with Neu5Aca2,3Galβ1,4GlcNAc/Glc trisaccharide [133, 134]. Mixtures of MAL and MAH can be used to detect glycans containing α-2,3 linked SA, and are the most interesting complementary probe to the SNA. These lectins combination can distinguish among different types of SA linkages and have been used as general tools in the detection, quantitation, localization, purification and characterization of many biomolecules containing SA. There are a few mushroom lectins reacting with Neu5Aca2,3Gal sequence too. *Agrocybe cylindracea* lectin (ACG) strongly interacts with glycoconjugates containing Neu5Aca2,3Galβ1,3GlcNAc/GalNAc sequences [135]. *Psathyrella velutina* lectin (PVL) and *Macrophomina phaseolina agglutinin* (MPA) exhibit high affinity towards sialoglycoproteins possessing trisaccharide α-2,3 linkages and as potential tools for separation and detection of sialoglycoproteins [136, 137].
<table>
<thead>
<tr>
<th>Lectin</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Wheat germ agglutinin</em> (WGA)</td>
<td>Neu5Ac, GlcNAc(β,1-4)GlcNAc</td>
</tr>
<tr>
<td><em>Sambucus nigra</em> lectin (SNA)</td>
<td>Neu5Ac(α-2,6)Gal/GalNAc</td>
</tr>
<tr>
<td><em>Polyporus squamosus</em> lectin (PSL)</td>
<td>Neu5Ac(α-2,6)Gal</td>
</tr>
<tr>
<td><em>Allomyrina dichotoma</em> lectin II (Allo A-II)</td>
<td>Neu5Acα2-6Galβ1-4GlcNAc</td>
</tr>
<tr>
<td><em>Maackia amurensis</em> hemagglutinin (MAH)</td>
<td>O-linked Neu5Ac(α-2,3)Gal</td>
</tr>
<tr>
<td><em>Maackia amurensis</em> leukoagglutinin (MAL)</td>
<td>N-linked or core 2 O-linked Neu5Ac(α-2,3)Gal</td>
</tr>
<tr>
<td><em>Agrocybe cylindracea</em> lectin (ACG)</td>
<td>Neu5Acα2,3Galβ1,3GlcNAc/GalNAc</td>
</tr>
<tr>
<td><em>Psathyrella velutina</em> lectin (PVL)</td>
<td>Neu5Acα2,3Galβ1,4GlcNAc, GlcNAc</td>
</tr>
<tr>
<td><em>Macrophomina phaseolina</em> agglutinin (MPA)</td>
<td>Neu5Ac(α-2,3)Galβ1,4GlcNAc</td>
</tr>
</tbody>
</table>
Fluorescent labeled lectins are wildly used in flow cytometry and fluorescent microscopy for research and diagnosis that related to SA alteration. For example, in one cancer research, MAL was used to investigate the expression levels of α-2,3 linked SA in 50 primary tumor cases, 50 pair-matched lymph node metastasis tumor samples and in the MDA-MB-231, T-47D and MCF-7 breast cancer cell lines with different metastatic potential. The expression of α-2,3 linked SA was analyzed by flow cytometry, histochemistry and cytochemistry after MAL labeling. As a result, the expression of α-2,3 SA showed a positive correlation with cancer cell metastatic potential, the higher α-2,3 linked SA level the stronger adhesion, invasion and migration activities [138]. Another study using same approach found high expression of α-2,3 linked SA was associated with the metastatic potential of human gastric cancer [139]. Moreover, with SNA labeling and flow cytometric detection, α-2,6 linked SA has been suggested to correlate with the adhesion of breast cancer cells [140, 141]. However, it should be considered that both α-2,3 and α-2,6 sialylation may play different roles in regulating cell adhesion of different cancer cells when developing potential therapeutics targeting for sialylation.

Furthermore, the strict specificity of lectins to glycan structures provides a valuable approach to identify the functional glycoconjugates. In a pilot study, a confocal microscopy method employed four different SA-specific lectins was established to successful identification and localization of cell surface and intracellular sialoglycoconjugates of peripheral blood cells [142]. Later, another study analyzed the expression of cell surface sialoglycoconjugates in Herpetomonas megaseliae by flow cytometry and fluorescence microscopy using lectins that specifically recognize SA residues [143]. Strong reaction was detected when parasites treated with slug Limax flavus lectin (LFA), MAA or SNA,
indicating that molecules containing α-2,3 and α-2,6 sialylgalactosyl sequences were present in this protozoan. Further study suggested a possible involvement of sialomolecules in the interaction between this insect trypanosomatid and the invertebrate host. Lectins also possess potential to selective detection of glycan sialylation on cancer cell surface. Recently, a study utilized two human mammary epithelial cell lines, HB4A (breast normal cells) and T47D (breast cancer cells) as a model system to demonstrate differential surface glycans when treated with SA under nutrient deprivation [144]. Although SA treatment of both cells resulted in increased activities of α-2,3/6 sialyltransferases, both flow cytometry and confocal microscopy showed a very strong staining on the membrane of SA-treated T47D cells, indicating an increase of Neu5Acα2,3Gal on the cell surface.

Although lectins have offered important approach to study cell surface sialylated structures, there are still several drawbacks. First, some of the plant lectins are glycosylated and it will complicate the results for study the complex samples that may contain endogenous lectins which could interact with the glycan of plant lectins [145]. In addition, many lectins can perturb cells by cross-linking receptors limiting their application in live cells [146]. At last, unlike other more detailed analysis by mass spectrometry and NMR, the lectins could only get a category glycan/glyoprotein and must draw assistance from the high resolution mass spectrometry to identify the structure for further study [147]. Nevertheless, lectins actually give a hand on the research of sialoglycoconjugates on cell surface and separation siaoglycoconjugates especially from complicated matrix in an affinity manner.
1.4.2 Free SA quantification

The SAs in different linkages on cell surfaces can be determined by flow cytometry and confocal microscopy after specific lectin labeling. However, different approach is required to analyze SA in other forms and systems. For example, the total amount of SA expressed by cells, and free SA portion in culture medium and human plasma. To answer these questions and to systematically study SA in different biological conditions, we introduced quantitative liquid chromatography coupled mass spectrometry (LC-MS/MS) to achieve accuracy and robust detection of SA in matrices, such as human plasma, cell culture medium, and cell lysate.

The special 9-carbon monosaccharide with one carboxylic acid structure provides no UV absorption, which initially vanished the possibility to directly quantify SA. Derivatization reagents with UV absorption such as resorcinol [148] and thiobarbituric acid [149] enables the detection of SA with colorimetric assay. In order to improve the detection sensitivity and specificity, new fluorometric methods were developed with modified thiobarbituric acid derivatization [150], or mild periodate oxidation coupled with Hantzsch reaction [151]. However, neither colorimetric nor fluorometric assays could differentiate SA from other interference, thus limiting their application in complex biological samples. Reversed-phase HPLC has the advantage of quantitatively differentiating various forms of SAs and limits the interference from matrix. For example, HPLC-UV with per-O-benzoylation has been reported to determine Neu5Gc and Neu5Ac in the culture media and cell-associated glycoconjugates from cancer cells [152]. However, the revolutionary advance of SAs quantification started after the development of quinoxaline derivatization followed by HPLC with fluorescence detection (Figure 5). Derivatization reagents such as
1,2-diamino-4,5-dimethoxybenzene (DDB) [153], o-phenylenediamine (OPD) [154], and 1,2-Diamino-4,5-methylenedioxybenzene (DMB) [155, 156] are initially reported to give strong fluorescent intensity. Among them DMB is the most sensitive one with high derivatization efficiency, thus it has been commonly used for SAs derivatization and also in commercial SAs quantification kits. However, both DMB reagent and its SAs derivative are very instable due to their light and oxygen sensitivity. Fresh DMB solution has to be prepared every day, which is time consuming and increases the cost for the expensive DMB reagent. In our lab we have reported two stable and cost-effective quinoxaline derivatization reagents, 3,4-diaminotoluene (DAT) [157] and 4,5-dimethylbenzene-1,2-diamine (DMBA) [158], both of which give high reaction efficiency and intense signal in fluorescence and mass spectrometry quantification.
Figure 5: Quinoxaline derivitization of SAs
The LC-MS/MS quantification coupled with quinoxalinone derivatization possesses high sensitivity and high resolution, which has provided a powerful tool for SAs analysis in the complex biology samples [159-161]. A very successful application is to study SA diversity, and identify small amount of unexpected SAs or new members of their family [160, 161]. One study used electrospray ionization-mass spectrometry (ESI-MS) to characterize SAs after the HPLC separation of their DMB derivatives from different biology source [160]. As a result, 28 different SAs including more than 10 new species were identified. Furthermore, this approach was able to characterize the sulfate esters of SAs such as Neu5Ac8S and Neu5Gc8S, which were not detected using other methodologies. Another similar study used LC-MS/MS to compare SA species and distributions in different tissues of rats and mice [161]. Results showed a wide variety of SAs existed in tissue-specific manner in mice and rats, and highly acetylated SAs and 8-O-sulfo-Neu5Ac were commonly distributed in all the organs examined. For example, mouse sera contained Neu5Gc as the major SA, but Neu5Ac and Neu5,9Ac2 were the major components in rat sera. Sublingual and submaxillary glands of rats showed wide distributions of various SAs, but those of mice contained simple SA composed of Neu5Ac as the major one. Although the reason different SAs are found in these tissues is not clear, different enzymes such as acetyl transferase and sulfotransferase play some significant roles.

The derivatization method indeed provides valuable tool to identify and quantify (if the standard compounds are available) SA species in various biology samples. However, the sample preparation is tedious and time consuming, which limits its application in large scale sample analysis, such as in clinical case. One of the most attractive area in clinical
application is the diagnosis of SA storage disease [162-164]. SA storage diseases (SSDs) are inborn error resulting from defects in the lysosomal membrane protein sialin, which leads to accumulation of SA in tissues, fibroblasts, and urine. Defective free SA transport can be established by quantification of free SA in urine. Direct SA quantification with LC-MS/MS has been reported to be more rapid, accurate, and sensitive than earlier methods for SA detection in the urine [162]. In one clinical study, free SA in the urine samples was separated by amino propyl guard column with gradient elution, and then detected by triple-quadrupole mass spectrometer in negative mode [162]. This method was used to quantify free SA in urine samples from 72 control people and 3 SSDs patients. The results showed this method can successfully distinguish the SSDs from the healthy individuals. For example, patients at 1.2 years old had SA of 111.5 mmol/mol creatinine, compared to the normal range 0.7-56.9 mmol/mol creatinine obtained from 20 normal people. In another clinical study, both urine free and total SA were analyzed by a newly developed LC-MS/MS approach using an Atlantis dC18 analytical column to separate SA from the interferences [163]. Free SA was analyzed directly after a simple filtration step, and total SA was obtained by one step acid hydrolysis. This method achieved a semi-automated sample preparation in combination with a runtime of 6 min, which made it suitable for high throughput analysis. Moreover, based on urine samples from 589 individuals and 11 SA metabolism deficient patients, age-related reference values for free SA, total SA and conjugated SA were determined and improved the diagnostic efficacy. Later a similar LC-MS/MS approach with small modification was used to determine free and conjugated SA in amniotic fluid supernatant (AFS), which is also used for SSDs screening test [164]. The whole procedures from sample preparation to MS analysis can be performed in 2-4 h.
Reference values in AFS were 0-8.2 μmol/L for 15-25 weeks of gestation and 3.2-12.0 μmol/L for 26-38 weeks of gestation. In AFS samples from five fetuses affected with infantile SSD free SA was 23.9-58.9 μmol/L demonstrating that this method was able to discriminate infantile SSD pregnancies from normal ones.
1.5 Reference


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CHAPTER II
QUANTIFICATION OF FREE SIALIC ACID IN HUMAN PLASMA
THROUGH A ROBUST QUINOXALINONE DERIVATIZATION
AND LC-MS/MS USING ISOTOPE-LABELED STANDARD
CALIBRATION FORMULATION

2.1 Introduction

SAs, a family of 9-carbon containing acidic monosaccharides, often terminate the
glycan structures of cell surface and secreted glycoconjugates such as glycoproteins and
glycolipids. SAs are involved in many biological processes like immunological process,
hormonal response, signal transmission, tumor progression, cell adhesion, and protection
mechanisms [1,2]. It has been demonstrated that cancers and cancer stages may be
associated with a significant overrepresentation of SAs on the glycoproteins of cancer cell
surface [3,4]. In addition, SAs exist in both free and conjugated forms in tissues and fluids
such as serum, urine and saliva. Also, it is known that the amount
of SAs in serum is elevated in cardiovascular diseases, and is a strong predictor of cardiovascular mortality [5,6]. Therefore, SAs have been considered as a biomarker for certain types of cancers, cardiovascular diseases and some other diseases.

Because of the great significance of SAs in physiological and clinical applications, several analytical methods have been developed for quantification of SAs, either free SAs or SAs released from glycoconjugates in variety of matrices. These include colorimetric [7,8] and fluorometric [9,10] assays, liquid chromatography coupled with different detection techniques, such as pulsed amperometric detection [11,12,19], UV [13], fluorescence [14-20] and mass spectrometry [21-31]. Sample preparation involves either non-derivatization [11,12,24-29] or derivatization [7-10, 13-23,30,31] before analysis. Among all these methods, DMB derivatization of SAs combined with HPLC fluorescent detection [15,16,18-20] and LC-MS/MS detection [22,31] has been the most frequently used assay due to its high specificity and sensitivity. DMB reacts with α-keto carboxylic acid, and provides a good response with both fluorescent and mass spectrometric detections. However, DMB is a highly light sensitive and expensive reagent, which has to be stored under -20 °C in dark with inert gas. Particular cautions must be taken in the derivatization procedure. Further, slight variations in the experiment conditions could lead to a poor reproducibility of the results. In order to overcome these limitations, other phenyldiamines such as OPD [17] and DDB [23] had been investigated with improved product stability. In this study, we have checked the chemical and physical properties of commercial available phenyldiamines and thoroughly investigated a much stable and inexpensive DAT for SAs derivatization and LC-MS/MS analysis (Figure 6).
Figure 6: SA is derivatized with DAT, and then quantified by LC-MS/MS.
In the FDA guidelines for validation of bioanalytical methods, the same biological matrix as the matrix in the intended samples should be used for validation purposes whenever possible. However, the presence of endogenous SAs in plasma causes a difficulty to construct matrix-matched calibration curve for accurate analysis. To solve this problem, the most adoptive ways use water [26-30] as matrix or standard addition calibration [21,23] in SAs quantification. The problem associated with water is that it cannot reflect the suppression or enhancement of signal caused by co-eluting components in complex biomatrices, especially with ESI-MS [32,33]. Although standard addition can avoid the matrix effect, the high background of SAs in biomatrices makes it hard to accurately calculate a small change from a large value. In this study, we used a stable isotope-labeled compound $^{13}$C$_3$-SA to prepare calibrators and quality control (QC) samples in plasma. In particular, isotope-labeled compounds possess great similarity in chemical properties with native compounds, and can provide true relationship between the detection response and the analyte concentration in a matrix. Overall, a good reproducibility, accuracy and sensitivity were achieved for free SAs quantification employing UHPLC-MS/MS and isotope-labeled standard calibration (ILSC) combined with DAT derivatization.
2.2 Experimental

2.2.1 Materials

N-Acetyl-D-neuraminic acid (SA, 98%) was purchased from Rose Scientific Ltd. (Edmonton AB, Canada), N-acetyl-D-neuraminic acid-1,2,3-\(^{13}\)C\(_3\) \(\text{\(^{13}\)C}_3\)-SA, 99%), ketodeoxynonulosonic acid (KDN, 99%), 3,4-diaminotoluene (DAT, 97%), 1,2-diamino-4,5-methylenedioxybenzene (DMB, 98%), 4-bromo-1,2-diaminobenzene (97%), 4-nitro-O-phenylenediamine (98%), 2-mercaptoethanol (99%), and sodium hydrosulfite (85%) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Acetonitrile (HPLC grade), methanol (HPLC grade) and acetic acid (ACS grade) were obtained from Fisher Scientific (Hanover park, IL, USA). Deionized water was generated from Barnstead Nanopure Water Purification System (Asheville, NC, USA). Human plasma for method validation was purchased from Innovative Research (Novi, MI, USA). Patient and normal plasma samples were obtained from Cleveland Clinic (Cleveland, OH, USA).

2.2.2 Stock and work solutions

DAT solution at 10.0 mM was prepared by dissolving DAT reagent in 1.4 M acetic acid containing 18 mM sodium hydrosulfite and 0.75 M 2-mercaptoethanol. The solution was kept in refrigerator at 4 °C.

The stock solution of \(^{13}\)C\(_3\)-SA was prepared in plasma at 1.00 mg/mL, then serially diluted with plasma to provide work standard solutions at 20.0, 60.0, 200, 600, 2.00 \(\times\) 10\(^3\), 6.00 \(\times\) 10\(^3\), 2.00 \(\times\) 10\(^4\) ng/mL, which were used as the calibrators. These work solutions for preparation of QC samples were generated in a similar manner at the concentrations of 40.0, 800, and 1.60 \(\times\) 10\(^4\) ng/mL. All the \(^{13}\)C\(_3\)-SA work solutions were aliquoted into 50
μL per vial. IS work solution was made by dilution of KDN with water to the concentration of $3.00 \times 10^3$ ng/mL. The stock and work solutions were stored at -20 °C until analysis.

### 2.2.3 Sample preparation

Protein precipitation (PPT) procedure was employed to extract free SA, $^{13}$C$_3$-SA and IS from plasma samples, including the calibrators, QC standards, blanks and the unknown samples. After plasma samples were thawed to room temperature, 10 µL of IS work solution was spiked into each sample of 50 µL except the blanks, in which 10 µL of water was used. Following a 30 seconds vortex, 150 µL of acetonitrile was added into each sample, which was vortexed for another 1 min, and then centrifuged at 15,000 g for 5 minutes. Afterwards, 20 µL of supernatant was transferred to another 1.5-mL centrifuge tube, and mixed with 180 µL DAT solution. The reaction was processed at 80 °C for 40 min, and stopped by cooling in ice water for 5 min. The derivatized samples were then analyzed using UHPLC-MS/MS as described below.

### 2.2.4 LC-MS/MS instrumentation and conditions

The UHPLC was a NexeraTM system, consisting of a DGU-20A3R degasser, two LC-30AD pumps, a SIL-30AC autosampler, a CTO-10A column oven and a CBM-20A system controller from Shimadzu (Columbia, MD, USA). The UHPLC system was interfaced to a Qtrap 5500 mass spectrometer equipped with eletrospray ionization source from AB SCIEX (Framingham, MA, USA). Data acquisition and processing were conducted using Analysis 1.6.1 software packaged with Multiquan from AB SCIEX.
For chromatographic separation, 10 μL of prepared samples were injected onto a Phenomenex Kinetex C18 column (2.1 × 50 mm, 1.7 μm) guarded with a C18 SecurityGuard ultra guard column (Torrance, CA, USA). The column temperature was maintained at 35 °C during the analysis. The mobile phase was a mixture of water-methanol (80/20, v/v) at an isocratic flow of 0.3 mL/min.

The MS detection employed positive electrospray ionization, and multiple reaction monitoring (MRM) for quantification. The quantitative MRM transitions were set at m/z 396 → 253 for SA, 399 → 256 for $^{13}$C$_{3}$-SA, and 355 → 175 for IS; the quality assurance MRM channels were 396 → 283 for SA and 399 → 286 for $^{13}$C$_{3}$-SA. The optimal MS parameters were as follows: Ion Spray Voltage 4500 V, Curtain Gas 30 psi, Nebulizer Gas 30 psi, Heating Gas 40 psi, and Source Temperature 550 °C.

### 2.2.5 Quantification

Quantification of free SA in plasma was performed using $^{13}$C$_{3}$-SA as calibration standard and KDN as IS. SA concentration in unknown sample was calculated according to the equation: $C = C_i \times 0.990$, where $C$ was the SA concentration; $C_i$ was the value for the sample obtained directly from the calibration curve, which was constructed using Multiquan by assaying $^{13}$C$_{3}$-SA spiked plasma calibrators at seven concentrations in the range of $20.0 - 2.00 \times 10^4$ ng/mL; 0.990 was the conversion factor, calculated by the ratio of molecular weight of SA to $^{13}$C$_{3}$-SA.
2.3 Results and discussion

2.3.1 Derivatization of SAs

SAs exist in either free form or conjugated form of glycoproteins and glycolipids. Among all the SA species, Neu5Ac is the most abundant in human. In this study, we aimed to develop a sensitive and accurate method for quantification of free Neu5Ac in human plasma. DMB-based quinoxalinone derivatization has been widely used for SA quantification [15, 16, 18-20, 22, 31], in which DMB is specialized for α-keto carboxylic acid with good florescent and MS response. However, DMB is not only instable due to light and oxygen sensitivity, but also very expensive. In our experiments, we observed that DMB changed color during a short weighing process, and the chromatographic peak area of the DMB-SA varied day to day. Therefore, fresh DMB solution needs to be prepared for each assay, which is very time consuming and often causes analytical variations due to the high instability of this reagent. In order to overcome this limitation, we have examined other commercially available compounds having the similar 1,2-diamino-benzene structure as DMB for SA derivitization and LC-MS/MS analysis (Figure 6). First, commercial free SA (Neu5Ac) was used for derivitization and optimization study. We found that SA derivatization with DAT afforded a similar MS intensity as DMB, while 4-bromo-1,2-diaminobenzene, and 4-nitro-O-phenylenediamine were about 1/10 and 1/100 of DMB response, respectively. Therefore, we chose DAT for further optimization of the derivatization conditions including the concentration, reaction time and temperature. As a result, DAT concentration higher than 5 mM gave the maximum and constant MS response, and 80 °C gave the most intense MS peak comparing to 50 °C and 37 °C reaction temperature. In addition, the highest MS response was achieved after 30 min, and then
began to decrease after 80 min. Based on these results, the derivatization of SA was set at 80 °C for 40 min with 10 mM of DAT.

For comparing the stability and reproducibility of DAT and DMB derivatives, the two freshly prepared reagents were reacted with standard SA solutions at 40 ng/mL individually on seven consecutive days in our newly developed procedure above and using the literature condition [16], in which DMB dihydrochloride (7.0 mM) was dissolved in 1.4 M acetic acid containing 18 mM sodium hydrosulfite and 0.75 M 2-mercaptoethanol and then the reaction was conducted at 50 °C for 2.5 h in dark, respectively. The derivatization products of each day were stored at -20 °C in dark, and analyzed at the end of the seventh day all together in one batch using LC-MS/MS. DMB-IS and DAT-IS samples were prepared on the seventh day, and spiked into each of the corresponding SA derivatives before LC-MS/MS analysis. As a result (Table III), DMB-SA showed a relative error from -37% to 88%, while DAT-SA had only -6% to 9% error. Since both reagents and their products were stabilized by sodium hydrosulfite and 2-mercaptoethanol, the larger variation of DMB-SA may be due to its high instability with light and oxygen, which makes it difficult to handle in a repeatable manner. Furthermore, another advantage of DAT was the low price, thousands of times cheaper than DMB. In fact, the high cost of DMB is a problem considering that its reagent solution needs to be prepared freshly each time.
<table>
<thead>
<tr>
<th>Spiked conc.</th>
<th>1st day</th>
<th>2ed day</th>
<th>3rd day</th>
<th>4th day</th>
<th>5th day</th>
<th>6th day</th>
<th>7th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMB-SA (ng/mL)</td>
<td>40.0</td>
<td>50.3</td>
<td>75.2</td>
<td>25.3</td>
<td>46.3</td>
<td>32.0</td>
<td>57.6</td>
</tr>
<tr>
<td>RE %</td>
<td>26</td>
<td>88</td>
<td>-37</td>
<td>16</td>
<td>-20</td>
<td>44</td>
<td>0</td>
</tr>
<tr>
<td>DAT-SA (ng/mL)</td>
<td>40.0</td>
<td>37.7</td>
<td>42.8</td>
<td>43.6</td>
<td>39.1</td>
<td>38.7</td>
<td>40.7</td>
</tr>
<tr>
<td>RE %</td>
<td>-6</td>
<td>7</td>
<td>9</td>
<td>-2</td>
<td>-3</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>
2.3.2 Isotope-labeled standard calibration (ILSC)

According to the FDA guidelines for validation of bioanalytical methods, the calibrators and QCs for analysis should be prepared using the same types of matrices as samples to be measured [34]. It has been strongly recommended to use matrix-matched calibration for quantification assays employing LC-MS or LC-MS/MS due to such a matrix effect that some of the matrix components could suppress or enhance the mass spectrometry signals of the analytes or IS [32]. An HPLC-ESI-MS/MS method developed for the quantification of free SA in urine employed creatinine-adjusted urine as blank matrix for calibration preparation, which had a limit of detection of 927 ng/mL [24]. In order to achieve highly sensitive and accurate quantification, we used $^{13}$C$_3$-SA as a surrogate standard to generate calibration curves and QC samples. Since $^{13}$C$_3$-SA possesses the same chemical structure and properties as SA, and they have identical stability, derivatization efficiency, chromatographic retention, and MS response and fragmentation pattern, the specific and sensitive detection of them could be obtained by mass spectrometry in MRM mode upon their difference in m/z values.

2.3.3 Optimization of LC-MS/MS conditions

Chromatographic conditions were optimized to obtain higher specificity, sensitivity, and shorter analysis time. Various mobile phases including different percentages of methanol in water and acetonitrile in water with or without additives were evaluated, and 20% methanol at 0.3 ml/min provided the optimal results. It was found that the LC-MS/MS signal from $^{13}$C$_3$-SA was at least two times higher with methanol-water as mobile phase than acetonitrile-water. Addition of formic acid or ammonium acetate into
the mobile phase substantially reduced the peak intensity. Under this selected chromatographic conditions, the retention times for SA, $^{13}$C$_3$-SA and IS were 3.6 min, 3.6 min, and 2.4 min, respectively. The total run time was 4.2 min, which was the shortest analysis time for quantification of free SA reported so far [11-31].

The product ion scan spectra of [M+H]$^+$ precursors from the derivatized SA, $^{13}$C$_3$-SA and IS are illustrated in Figure 7 A-C; the proposed fragmentation pathway for SA is shown in Figure 8. The calibration standard, $^{13}$C$_3$-SA, had three relatively abundant fragments with m/z 256, 286, and 381. However, m/z 256 was found to have the highest signal to noise ratio (S/N) at LLOQ concentration than the other two fragment ions. After optimization and evaluation, we selected transitions of m/z 396 $\rightarrow$ 253 for SA, 399 $\rightarrow$ 256 for $^{13}$C$_3$-SA, and 355 $\rightarrow$ 175 for IS as the MRM channels for quantification. The transitions of 396 $\rightarrow$ 283 for SA and 399 $\rightarrow$ 286 for $^{13}$C$_3$-SA were also monitored during LC-MS/MS analysis for quality assurance.
Figure 7:  Product ion spectra of $[\text{M+H}]^+$ precursors. (A) DAT-SA, (B) DAT-$^{13}\text{C}_3$-SA, (C) DAT-IS.
Figure 8: Proposed fragmentation pathway of [DAT-SA+H]^+. 
2.3.4 Free SA extraction from plasma

There are both free SA and conjugated SA in human plasma, all together called total SA, and free SA accounts a small portion of total SA. Therefore, it is critical to effectively extract free SA from the plasma and separate the conjugated forms from the extractants. In this study, three different extraction methods were examined, including PPT with acetonitrile and methanol, and dialysis with 3 kD centrifugal filter (Amicon® Ultra, catalog number R1NA37692). Methanol and acetonitrile were often used for PPT with human fluid to obtain free SA [28,29]. In our study, free SA peaks in plasma samples after methanol-PPT were about 30 times higher than those after acetonitrile-PPT (Table IV). This may be attributed to the incomplete removal of conjugated SA from plasma. To test this hypothesis, the supernatants of both methanol- and acetonitrile-PPT samples were dried with nitrogen at 37 °C, and the residues were reconstituted into the same volume of water. Then 20 μL of each sample was subjected to 15% SDS-PAGE analysis. As shown in Figure 9, trace proteins exist in methanol-PPT samples, while no protein bands were observed in acetonitrile-PPT samples, indicating a complete remove of proteins. Therefore, acetonitrile-PPT was used to remove the conjugated SA and obtain the free SA from plasma in our study.
TABLE IV: SA peak areas from different sample preparations (n = 3).

<table>
<thead>
<tr>
<th></th>
<th>Dialysis</th>
<th>Methanol-PPT</th>
<th>Acetonitrile-PPT</th>
<th>Non-extraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Area (counts)</td>
<td>((6.31 \pm 0.42) \times 10^5)</td>
<td>((1.69 \pm 0.13) \times 10^7)</td>
<td>((6.02 \pm 0.24) \times 10^5)</td>
<td>((2.54 \pm 0.21) \times 10^8)</td>
</tr>
</tbody>
</table>

Figure 9: SDS-PAGE chromatograms of plasma and its PPT samples: (A) Protein ladder, (B) human plasma, (C) plasma samples precipitated with methanol, (D) plasma samples precipitated with acetonitrile.
2.3.5 Method validation

2.3.5.1 Linearity of calibration

Calibration curves were constructed using seven calibrators prepared from pooled plasma spiked with \(^{13}\text{C}_3\)-SA at concentration range of \(20.0 - 2.00 \times 10^4\) ng/mL and IS at \(3.00 \times 10^3\) ng/mL. A typical equation obtained from weighted (1/x) linear regression was \(y = 9.27 \times 10^{-4}x - 3.13 \times 10^{-4}\), where \(y\) was the peak area ratio of \(^{13}\text{C}_3\)-SA to IS, and \(x\) was the concentration of \(^{13}\text{C}_3\)-SA in spiked plasma. The correlation coefficient (r) was at least 0.999, indicating that the calibration was highly linear within the concentration range.

2.3.5.2 Specificity, sensitivity, and LLOQ

The specificity and sensitivity were evaluated by analyzing LLOQ samples prepared from six individual lots of plasma and the blanks (no \(^{13}\text{C}_3\)-SA or IS) from the same lots of plasma. As shown in Figure 10, no significant interference was observed in the retention time windows of \(^{13}\text{C}_3\)-SA and IS. The coefficient of variation (CV) and relative error (RE) for measurement of LLOQ samples were 6% and -1%, respectively, which indicated that this assay was sensitive enough to quantify SA at 20 ng/mL.
Figure 10: Representative MRM chromatograms of native/labeled SA and IS. (A) A pooled blank plasma sample, (B) a pooled plasma sample spiked with $^{13}\text{C}_3$-SA at the LLOQ of 20.0 ng/mL and IS, (C) a plasma sample from a patient spiked with IS.
2.3.5.3 Extraction recovery and matrix effect

The extraction recovery of free SA from plasma using acetonitrile-PPT was determined by comparing chromatographic peak areas from two sets of plasma samples. The first set was spiked with $^{13}$C$_3$-SA at three QC levels and IS before PPT, and the second set was spiked at the corresponding concentrations after PPT. The absolute extraction recovery of $^{13}$C$_3$-SA was over 70%, and the IS-normalized recovery was nearly 100%. To evaluate the matrix effect, the peak areas obtained from the second set of extraction recovery samples were compared with those of $^{13}$C$_3$-SA and IS prepared in water at equivalent concentrations. Both the absolute and IS-normalized matrix effect were close to 100%, which meant the matrix effect was negligible in this study.

2.3.5.4 Precision and accuracy

The intra-assay and inter-assay precision and accuracy were studied by determining the QC samples at three concentrations in five replicates, and the results are listed in Table V. The CV was within 5% and the RE was in the range of -5 to 5%, which well fulfilled the FDA criteria for bioanalysis [34].
TABLE V: Intra- and inter-assay precision and accuracy for measurement of spiked $^{13}$C$_3$-SA in plasma (n=5)

<table>
<thead>
<tr>
<th>QC</th>
<th>Spiked conc. (ng/mL)</th>
<th>Intra-assay</th>
<th>Inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean ± SD (ng/mL)</td>
<td>Precision (CV %) $^a$</td>
</tr>
<tr>
<td>Low</td>
<td>40.0</td>
<td>39.9 ± 1.4</td>
<td>3</td>
</tr>
<tr>
<td>Mid</td>
<td>800</td>
<td>839 ± 17</td>
<td>2</td>
</tr>
<tr>
<td>High</td>
<td>$1.60 \times 10^4$</td>
<td>$(1.67 ± 0.03) \times 10^4$</td>
<td>2</td>
</tr>
</tbody>
</table>

$^a$ CV % = (SD/Mean) × 100%

$^b$ RE % = [(Mean – Spiked)/Spiked] × 100%
2.3.5.5 Stability

The stability of SA-DAT derivatives in human plasma was assessed by analyzing five replicates of low and high QC samples undergoing different storage conditions pre and post sample preparations. As shown in Table VI, there was essentially no change with $^{13}$C$_3$-SA in the QC samples kept at -80 °C for two weeks, or room temperature (RT) for 8 hours. It was also stable in the autosampler at 4 °C for 10 hours post sample preparation.
TABLE VI: Stability of $^{13}$C$_3$-SA in plasma pre and post sample preparation ($n = 5$)

<table>
<thead>
<tr>
<th>Storage condition</th>
<th>QC</th>
<th>Spiked conc. (ng/mL)</th>
<th>Mean ± SD (ng/mL)</th>
<th>Stability (Recovery %) $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 weeks at -80 °C</td>
<td>Low</td>
<td>40.0</td>
<td>41.1 ± 2.8</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>$1.60 \times 10^4$</td>
<td>$(1.60 \pm 0.06) \times 10^4$</td>
<td>100</td>
</tr>
<tr>
<td>3 freeze (-80 °C)-thaw (RT) cycles</td>
<td>Low</td>
<td>40.0</td>
<td>42.3 ± 1.5</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>$1.60 \times 10^4$</td>
<td>$(1.70 \pm 0.06) \times 10^4$</td>
<td>106</td>
</tr>
<tr>
<td>8 h at RT</td>
<td>Low</td>
<td>40.0</td>
<td>39.8 ± 1.9</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>$1.60 \times 10^4$</td>
<td>$(1.68 \pm 0.06) \times 10^4$</td>
<td>105</td>
</tr>
<tr>
<td>10 h at 4 °C post preparation</td>
<td>Low</td>
<td>40.0</td>
<td>42.6 ± 1.3</td>
<td>107</td>
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<tr>
<td></td>
<td>High</td>
<td>$1.60 \times 10^4$</td>
<td>$(1.75 \pm 0.04) \times 10^4$</td>
<td>110</td>
</tr>
</tbody>
</table>

$^a$ Recovery % = Mean/Spiked × 100%
2.3.6 Assay application

This validated method was applied to determine free SA in plasma samples from 30 patients and 6 normal people. The free SA concentration in patient samples ranged from 419 to 862 ng/mL with an average of 651 ng/mL, while in normal people, the free SA concentration ranged from 280 to 354 ng/mL with an average 323 ng/mL (Figure 11). To our best knowledge, this is the first study to quantify free SA in human plasma using LC-MS/MS. In order to make statistical assumption, measurement of a large number of samples needs to be carried out in the future.
Figure 11: Free SA in plasma samples from 30 patients and 6 healthy people.
2.4 Conclusion

We developed a LC-MS/MS method with isotope-labeled standard calibration for sensitive quantification of free SA in human plasma, in which free SA, $^{13}$C$_3$-SA as calibration standard, and KDN as IS were derivatized with a stable and inexpensive DAT. Briefly, plasma samples were first mixed with acetonitrile to precipitate proteins and separate free SA from conjugated SA. Then the free SA, $^{13}$C$_3$-SA, and IS were derivatized with DAT. The derivatives were injected onto a Phenomenex Kinetex C18 column for separation using a mixture of water-methanol (80/20, v/v) as the mobile phase at an isocratic flow of 0.3 mL/min. The detection was performed on an AB Sciex Qtrap 5500 mass spectrometer with positive ESI employing multiple reaction monitoring (MRM) mode. Overall, we have demonstrated a robust derivatization of SA with DAT, which is cost-effective and provides a stable product of SA with high MS response. We have achieved a LLOQ of 20 ng/mL and a total run time of 4.2 min, which is the most sensitive and fast measurement for free SA in biomatrices. This assay was validated for linearity, precision and accuracy, and stability following the FDA guidance for bioanalytical method validation. The method was successfully applied to quantification of free SA in human plasma and is expected to be applied for accurate quantification of SA for both biological research and clinical applications.
2.5 References


CHAPTER III
GLOBALLY PROFILING SIALYLATION STATUS OF
MACROPHAGES UPON STATIN TREATMENT

3.1 Introduction
SAs ubiquitously distribute on the cell surfaces and locate on the outermost position of the glycan structures in a variety of glycoconjugates [1-3]. This particularly spatial orientation and great abundance endows SAs with important functions in a wide range of physiological and pathological processes, such as immunological process, cell adhesion and infection [3, 4]. SAs expressed on immune cells are closely related to their activities. An early study found that macrophages form rosettes with normal sheep erythrocytes but not the one after sialidase pretreatment [5]. Also, guinea pig peritoneal macrophages pretreated with sialidase bound more 125I-IgG than non-treated cells [6]. Further, sialidase treatment can substantially enhance the capacity of resting B cells to stimulate the proliferation of allogeneic and antigen specific syngeneic T cells [7-9]. All these studies reveal a fact that cell surface SAs on immune cells are involved in a diverse regulation of
immune cell interactions and play important roles related to both physiological and pathological processes.

So far, a number of studies have been performed on the SA binding proteins in the immune system. The SAs recognizing proteins are categorized to the family of siglecs, which represents SAs recognizing Ig superfamily lectins. The founding members of the siglec family include sialoadhesin (siglec-1), a macrophage adhesion molecule [10-12]; CD22 (siglec-2), a B-cell inhibitory receptor [13]; CD33 (siglec-3), a marker of myeloid cells [14]; and myelin associated glycoprotein (MAG or siglec-4), expressed by oligodendrocytes and Schwann cells in the nervous system [15]. Despite the extensive studies on the SAs binding proteins in the immune system, the SAs profile of immune cells themselves was largely overlooked for decades. In particular, the sialylation status related to the linkages and the amount of SAs on the immune cell surface is less known so far.

It was reported that cell surface sialylation pattern changes during apoptosis in several cell types, including colon carcinomas and thymocytes [16, 17]. Also, cell surface sialylation plays a role in modulating sensitivity towards APO-1-mediated apoptotic cell death [18]. A recent study found that decreasing of SA residues on the surface of apoptotic lymphocytes was recognized as an “Eat-Me” signal [19]. In addition, it was reported that ST6Gal-1 regulates macrophage apoptosis via α-2,6 sialylation of the TNFR1 death receptor [20].

Statin drugs, as inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, are widely used in the treatment of hypercholesterolemia for more than a decade. In addition to their lipid-lowering effect, convincing evidence demonstrated that statins have a wide effect on macrophages. For example, statins were found to reduce the
cell numbers of macrophages in vivo and in vitro [21-23], and one mechanism could be attributed to its apoptosis induction effect on macrophages at even low dosages [22, 23]. Moreover, the immunomodulatory roles of statin have been studied in macrophages on the cytokine and corresponding protein levels, such as inhibiting inducible nitric oxide synthase [24, 25], augmenting LPS-induced proinflammatory responses [26], and inducing Th2 cytokines secretion while suppressing Th1 cytokines production [27]. Although detail mechanisms have been elucidated in each study, most of these pleiotropic effects are mediated by the inhibition of the synthesis of isoprenoid intermediates such as farnesylpyrophosphate and geranylgeranylpyrophosphate, which are essential for many cellular functions like differentiation and proliferation [28].

In this study, we systematically investigated the sialylation status of macrophages in the normal culture condition and after atorvastation treatment. In particular, we globally profiled SAs linkages and levels of respective SA forms on the cell surface by confocal microscopy and flow cytometry with SA-specific lectins SNA and MAA. Further, free, conjugated, and total SAs in the cell culture medium and total SAs in the cells were quantified in both normal and statin-treated conditions by a newly developed LC-MS/MS method. The combination of confocal microscopy, flow cytometry, and LC-MS/MS provides the first time of global investigation of the cellular sialylation status of macrophages in the entire cell culture system, which will contribute to a better understanding of the physiological and pathological roles of SAs in macrophages and in the immune system as well.
3.2 Materials and methods

3.2.1 Materials

FITC labeled MAA lectin was purchased from bioWORLD (Dublin, OH, USA). FITC labeled SNA lectin was provided by Vector Laboratories (Burlingame, CA, USA). Alexa Fluor 488 labeled Annexin V, APC labeled Annexin V, and ProLong Gold Antifade Mountant were obtained from Life Technologies (Grand Island, NY, USA). Atorvastatin was purchased from A Chem Tek Inc. (Worcester, MA, USA). N-acetyl-D-neuraminic acid (SA, 98%) was supplied by Rose Scientific Ltd. (Edmonton AB, Canada). N-acetyl-D-neuraminic acid-1,2,3-13C₃ (13C₃-SA, 99%), 4',6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), RIPA buffer, protease inhibitor cocktail, rabbit anti-ST6Gal-I antibody, rabbit anti-ST6GalNAc-II antibody, rabbit anti-ST6GalNAc-VI antibody, and goat anti-rabbit IgG-peroxidase antibody were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Acetonitrile (HPLC grade), paraformaldehyde (PFA, 16% w/v), and acetic acid (ACS grade) were obtained from Fisher Scientific (Hanover park, IL, USA).

3.2.2 Apparatus

Flow cytometry was performed on a FACSCanto II system consisting of a blue and a red laser, operated through BD FACSDiva software (BD Bioscience, Mountain View, CA, USA). Images were acquired at a 60 × magnification using a Nikon A1Rsi confocal microscope, and data analysis was performed using NIS-Elements software (Nikon Instruments Inc., Melville, NY, USA). LC-MS/MS quantification was carried out by a Nexera liquid chromatography system (Shimadzu, Columbia, MD, USA) interfaced to a Qtrap 5500 mass spectrometer equipped with electrospray ionization source (AB
SCIEX, Framingham, MA, USA), and the data acquisition and processing were conducted using Analysis 1.6.1 software.

### 3.2.3 Cell culture and stimulation

Raw 264.7 cells, obtained from American Type Culture Collection, were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Cleveland Clinic Core Facility, Cleveland, OH, USA) supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Flowery branch, GA, USA) and 1% penicillin/streptomycin (Cleveland Clinic Core Facility, Cleveland, OH, USA) in a humidified atmosphere containing 5% CO₂ at 37 °C. In order to quantify free SAs, FBS was removed from culture medium before adding drug. For atorvastatin simulation, cells were seeded in 6-well plates for overnight, followed by atorvastatin treatment at different concentrations and incubation times. The media without FBS were collected and centrifuged at 15,000 g for 5 min. The supernatants were stored at -20 °C until use. Cells were analyzed by flow cytometry, confocal microscopy, or LC-MS/MS.

### 3.2.4 Flow cytometric analysis

For determination of cell surface SA, cells were treated with atorvastatin (or vehicle) for 24h. The cells were harvested and washed 3 times with cold phosphate buffered saline (PBS), and then suspended in 50 μL MAA (10 μg/mL) or SNA (20 μg/mL), and 5 μL PI (1000 μg/mL). After incubation for 30 min at room temperature, cells were washed with cold PBS for 3 times and re-suspended in the same buffer for flow analysis. A minimum of 20,000 cells were measured each time.
Cell apoptosis study was performed by staining cells with Annexin V and PI using the dead cell apoptosis kit (Life technologies, Grand Island, NY, USA). Briefly, cells treated with or without 20 μM atorvastatin for 14, 24, and 38 h were collected and washed 3 times with cold PBS, and then resuspended in 100 μL of 1 × binding buffer provided by the manufacturer. Annexin V (5 μL) and PI (1 μL) were added to the cell suspension and incubated at room temperature for 15 min. After incubation, 400 μL of 1 × binding buffer was added to each vial and cells were analyzed immediately with flow cytometry.

3.2.5 Confocal microscopy

Cells were seeded on cover slip in 6-well plate for overnight, and then treated with 20 μM atorvastatin (or vehicle) for 24 h. After incubation, cells on cover slip were washed for 3 times with cold PBS, and fixed with 4% PFA for 10 min. Excess PFA was removed by another 3 times wash with cold PBS. Then cells were stained with MAA-FITC (10 μg/mL) or SNA-FITC (20 μg/mL) for 30 min, followed by 1 min DAPI stain. Unbound lectin was washed off with cold PBS and cover glasses were mounted on ProLong Gold Antifade reagent.

3.2.6 LC-MS/MS analysis

SA and IS were separated by a Primesep D column (2.1 × 100mm, 5 μm; SIELC Technologies, Prospect Heights, IL, USA) with a binary linear gradient elution, in which phase A was deionized water containing 10 mM ammonium formate and 0.1% formic acid, and phase B was a mixture of 90% acetonitrile, 10% water, 3 mM ammonium formate, and 0.04% formic acid. The gradient program started at 10% B and maintained for 1 min, and
then changed linearly to 100% B in 3 min and maintained for 2 min. In order to equilibrate the column, the gradient sharply dropped to 10% B in 0.1 min and kept for 2.9 min. The total run time was 9 min for each sample. The MS detection was carried out in negative electrospray ionization and MRM mode. The quantitative MRM transitions were set at m/z 308 → 87 for SA, and 311 → 90 for IS. The quality assurance MRM channel for SA was set at m/z 308 → 170.

3.2.6.1 Method validation sample preparation

SA calibration standards were prepared in DMEM at concentrations of 2.00, 6.00, 20.0, 60.0, 200, 600, 2.00×10³ ng/mL. The QC samples were prepared in the same manner at low, mid and high concentrations of 4.00, 100, and 1.60×10³ ng/mL. IS was prepared in DMEM at the concentration of 2.00×10³ ng/mL. For the sample preparation, 5 μL IS was mixed together with 95 μL calibration standards or QC samples, and 5 μL of the mixture was subjected to the LC-MS/MS analysis.

3.2.6.2 Cell culture sample preparation

Free SA in the medium was quantified by spiking 95 μL of culture medium with 5 μL of IS, and then 5 μL of the mixture was injection to LC-MS/MS system. In order to determine total SA in culture medium, the sample was first hydrolyzed with 2 M acetic acid (1:1) at 80 °C for 90 min, and then 5 μL of the IS was added to 95 μL of the lysate before analysis. For quantification of total SA of the cell, the sample at a density of 1×10⁶ cells/mL was untrosonicated to obtain homogeneous mixture. Then follow the procedure of total SA quantification in culture medium.
3.2.7 Western blot

Cells were washed twice with ice-cold PBS and lysed with RIPA buffer on ice for 5 min. The cell lysates were collected and clarified by centrifugation at 15,000 g at 4 °C for 10 min. Protein concentrations were measured by the Bradford method using a protein assay kit (Bio-Rad, Hercules, CA, USA). Proteins (30 μg/sample) were fractionated on SDS-15% polyacrylamide gels and transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat milk in Tri-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature, and then probed with primary antibodies in TBS-T buffer with 5% nonfat milk at 4 °C overnight. The membranes were washed 3 times with TBS-T buffer, followed by incubation with secondary antibody for 1 h at room temperature. Antibody detection was accomplished by using the ECL Prime kit (Pierce) with the LI-COR Odyssey Fc imaging system.
3.3 Results

3.3.1 Cell surface SAs analyzed by confocal microscopy and flow cytometry

SAs are commonly found α-2,3 and α-2,6 linked to galactose residue on the cell surface [29]. In our study, we first performed confocal microscopy and flow cytometry analysis to distinctively measure SAs with different linkages on the macrophage surfaces by using MAA-FITC and SNA-FITC, which specifically bind to α-2,3 and α-2,6 linked SAs, respectively. From the confocal microscopy study, MAA-FITC showed very strong binding compared to SNA-FITC on the cell surface of macrophages cultured under the normal condition (Figure 12A). After cells were treated with atorvastatin for 24 h, MAA-FITC binding remained unchanged (Figure 12B), while SNA-FITC binding increased dramatically (Figure 12B).
Figure 12: Confocal microscopy analysis of cell surface SA. (A): Raw 264.7 cells at the normal culture condition were stained with MAA-FITC (10 μg/mL) and SNA-FITC (20 μg/mL), respectively. DAPI was used to stain nuclei. (B): Raw 264.7 cells were treated with 20 μM atorvastatin for 24 h followed by staining with lectins and DAPI. The scale bar represents 10 μm. (C): The fluorescent intensity of the confocal images was analyzed by ImageJ obtained from National Institutes of Health (n = 4).
Next, the lectin-labeled cells were analyzed by flow cytometry. The similar binding pattern was observed from macrophages under the normal and atorvastatin conditions. In particular, SNA-FITC binding for macrophages was significantly increased upon atorvastatin treatment (Figure 13). The dot plots indicated that approximately 80% of the cells were MAA positive and 10% were SNA positive in the normal condition (Figure 13A). After 24 h atorvastatin treatment, no apparent change in MAA-FITC labeling was observed, but peak shift was apparently found from SNA-FITC labeled cells (Figure 13B). The dot plots showed the total ratio of SNA positive cells increased about 3 times as compared with the untreated cells. Further, the lectin stainings were performed on permeabilized cells, and the peak shift was found in SNA-FITC labeled cells in both permeabilized and non-permeabilized conditions with atorvastatin treatment, while no significant change in MAA-FITC labeled cells were observed. Interestingly, large peak shift was found in SNA-FITC labeled cells with permeabilization independent on atorvastatin treatment (Figure 14). These results indicate that 2,6 linked SA increased both on the cell surfaces and inside the cells.
Figure 13: Determination of cell surface SAs by flow cytometry. (A): Raw 264.7 cells at normal condition were stained with MAA-FITC (10 μg/mL) and SNA-FITC (20 μg/mL), respectively. PI staining was used to distinguish living cells and dead cells. (B): Raw 264.7 cells were treated with 20 μM atorvastatin for 24 h then stained with lectins and PI. Data is a representative of at least three independent experiments.
Figure 14: Comparison of SAs level after cell permeabilization. Atorvastatin +: Cells were treated with 20 μM atorvastatin for 24 h. Atorvastatin -: Cells were incubated with vehicle for 24 h. Permeabilization +: Cells were fixed with 4% PFA, followed by permeabilization with 0.2% triton X-100, and then labeled with lectins. Permeabilization -: Cells were fixed with 4% PFA, and then labeled with lectins. (A): Raw 264.7 cells at different conditions were labeled with MAA-FITC (10 μg/mL). (B): Raw 264.7 cells at different conditions were labeled with SNA-FITC (20 μg/mL).
3.3.2 Comparison of SNA and Annexin V staining

It is known that statins stimulate apoptotic cell death in several types of immune cells, such as macrophages [22, 23]. Annexin V specifically binds to membrane phosphatidylserine and has been widely used for early apoptosis detection [30, 31]. In our study above, a significant SNA-FITC staining increase was observed for atorvastatin treated cells compared with MAA-FITC staining (Figure 12B and 13B). Therefore, we compared the SNA labeling and Annexin V labeling of the cells treated with 20 μM atorvastatin for 14, 24, and 38 h, respectively. As shown in Figure 15A, both SNA and Annexin V positive cells increased dramatically with the incubation time prolonged. Briefly, in the control sample (0 h), the SNA positive cell ratio was only 9.4%, which included both PI positive and negative cells, while the SNA positive cells increased to 23.5%, 33.6% and 41.3% when the cells were incubated with atorvastatin for 14, 24 and 38 h, respectively. Meanwhile, Annexin V positive cells increased in the same time-dependent manner as SNA labeling. However, the change scale of Annexin V labeling was smaller compared with SNA labeling. These results are further supported by the SNA and Annexin V co-stainings of the cells (Figure 15 B). All these results indicated that α-2,6 linked SAs were highly expressed on the surface of cells undergoing apoptosis and thus may be used as a marker for apoptosis detection as well. Further study to prove the correlation of macrophage apoptosis and α-2,6 linked SAs status is much deserved.
Figure 15: Comparison of SNA and Annexin V labeling of Raw 264.7 cells after cells incubated with atorvastatin. (A): Cells were treated with or without atorvastatin (20 μM) for 14, 24 and 38 h, and then labeled with SNA or Annexin V. Both PI positive and negative cells are included here. Values represent means and standard deviations from three independent experiments (n=3). (B): Cells were treated with atorvastatin (or vehicle) for 24h, and then co-stained with SNA-FITC and Annexin V-APC.
3.3.3 LC-MS/MS quantification of SAs in the culture media and cell lysates

3.3.3.1 Method validation

In this study, we developed a new LC-MS/MS method to quantitatively examine the free SAs and conjugated SAs levels in the cell culture media, and the total SAs in the cell lysates upon cell treated with atorvastatin. The mass spectra for both SA and $^{13}$C$_3$-SA (internal standard, IS) were obtained in negative electrospray ionization (Figure 16). The MS detection was carried out in negative electrospray ionization and multiple reaction monitoring (MRM) mode. The representative chromatograms for the LLOQ sample and a sample obtained from cells are shown in Figure 17. The calibration range was obtained from 2.00 to 2.00 $\times$ 10$^3$ ng/mL and the LLOQ was 2.00 ng/mL. Importantly, the large calibration range and low LLOQ facilitate the determination of small amount of free SAs in the cell culture medium and total SAs in the cell lysate. This method was validated for matrix effect (Table VII), precision and accuracy (Table VIII), and stability (Table IX).
Figure 16: The mass spectra of SA and $^{13}\text{C}_3$-SA (IS). SA or $^{13}\text{C}_3$-SA standard solution at a concentration of 100 ng/mL in water was infused into mass spectrometer at a speed of 10 μL/min. After ionization and fragmentation, the spectra were obtained from a product ion scan mode from m/z 50 to m/z 350. The fragment ions of m/z 87 for SA and m/z 90 for $^{13}\text{C}_3$-SA were selected for quantification. Fragment of m/z 170 for SA was used for quality assurance.
Figure 17: Representative MRM chromatograms of SA and $^{13}$C$_3$-SA. (A) a culture free DMEM medium sample, (B) a culture free DMEM medium sample spiked with SA at the LLOQ of 2.0 ng/mL and $^{13}$C$_3$-SA at 100 ng/mL, (C) a cell lysate sample from a cell density of $1 \times 10^6$ cells/mL spiked with $^{13}$C$_3$-SA at 100 ng/mL.
<table>
<thead>
<tr>
<th>Spiked SA (ng/mL)</th>
<th>Peak Area (counts)</th>
<th>Matrix Effect $^a$</th>
</tr>
</thead>
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<tr>
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<td>SA in Medium</td>
<td>SA in Water</td>
</tr>
<tr>
<td>4.00</td>
<td>$1.72 \times 10^4$</td>
<td>$1.72 \times 10^4$</td>
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<tr>
<td>100</td>
<td>$4.07 \times 10^5$</td>
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<tr>
<td>$1.60 \times 10^3$</td>
<td>$6.92 \times 10^6$</td>
<td>$6.59 \times 10^6$</td>
</tr>
</tbody>
</table>

$^a$ Matrix Effect = mean peak area of SA in culture medium / mean peak area ratio of SA in water
TABLE VIII: Intra- and inter-assay precision and accuracy for measurement of spiked SA in cell culture medium (n=5)

<table>
<thead>
<tr>
<th>Spiked SA (ng/mL)</th>
<th>Intra-assay</th>
<th>Inter-assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Precision (CV %)</td>
</tr>
<tr>
<td>4.00</td>
<td>3.90 ± 0.08</td>
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</tr>
<tr>
<td>100</td>
<td>97.9 ± 2.8</td>
<td>3</td>
</tr>
<tr>
<td>$1.60 \times 10^3$</td>
<td>$(1.65 ± 0.04) \times 10^3$</td>
<td>2</td>
</tr>
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</table>

*a CV % = (SD/Mean) × 100%

*b RE % = [(Mean - Spiked)/Spiked] × 100
<table>
<thead>
<tr>
<th>Storage Condition</th>
<th>Spiked SA (ng/mL)</th>
<th>Mean ± SD (ng/mL)</th>
<th>Stability (Recovery % )a</th>
</tr>
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<tr>
<td>8 h at RTb</td>
<td>4.00</td>
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<td>98.8</td>
</tr>
<tr>
<td></td>
<td>1.60 × 10³</td>
<td>(1.60 ± 0.01) × 10³</td>
<td>100</td>
</tr>
<tr>
<td>24 h at 4°C</td>
<td>4.00</td>
<td>3.96 ± 0.13</td>
<td>99.1</td>
</tr>
<tr>
<td></td>
<td>1.60 × 10³</td>
<td>(1.67 ± 0.06) × 10³</td>
<td>104</td>
</tr>
<tr>
<td>3 freeze-thaw (RT) cycles</td>
<td>4.00</td>
<td>4.07 ± 0.20</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>1.60 × 10³</td>
<td>(1.60 ± 0.03) × 10³</td>
<td>100</td>
</tr>
<tr>
<td>72h at 37°C</td>
<td>4.00</td>
<td>4.04 ± 0.17</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>1.60 × 10³</td>
<td>(1.60 ± 0.04) × 10³</td>
<td>100</td>
</tr>
</tbody>
</table>

a Recovery % = Mean/Spiked × 100%

b RT: room temperature
3.3.3.2 SAs concentration in cell culture samples

The validated method was successfully used for quantifying the free SAs and conjugated SAs in the cell culture media, and the total SAs in the cell lysates, which would indicate SAs releasing and synthesis fact related to cell function alteration (Figure 18). First, the concentrations of free SAs in the cell culture media under the normal culture condition and incubation with atorvastatin for 24 h were measured by LC-MS/MS. As a result, free SAs in the normal culture condition were 6.31 ± 0.45 ng/mL and no apparent change was observed after atorvastatin treatment. Meanwhile, the total SAs and conjugated SAs in the cell culture media were 73.2 ± 3.3 ng/mL and 66.9 ± 3.6 ng/mL under the normal condition and slightly increased to 80.9 ± 7.1 ng/mL and 74.3 ± 7.0 ng/mL, respectively, after incubation with 20 μM atorvastatin. Next, the total cellular SAs, including on the cell surfaces and inside the cells were measured by LC-MS/MS after releasing SAs from the cell lysates. As a result, the cellular SAs concentration was 369 ± 29 ng/mL at a density of 1×10^6 cells/mL under the normal culture condition, while it increased to 567 ± 25, 775 ± 32 and 1080 ± 50 ng/mL corresponding to 5, 10 and 20 μM of atorvastatin used, respectively.
Figure 18: SAs concentrations in the cell culture system. Cells were treated with vehicle (0 μM) or atorvastatin at 5, 10, and 20 μM for 24 h. Then free SAs in the medium were quantified by LC-MS/MS directly. The medium was hydrolyzed with acetic acid followed by LC-MS/MS analysis to determine the total SAs. Conjugated SAs in the medium were calculated by subtracting the free form from total SAs. In order to quantify total SAs of the cell, cells were first ultrasonicated to obtain homogeneous mixture, followed by acid hydrolysis and LC-MS/MS analysis. MF, medium free SA; MC, medium conjugated SA; MT, medium total SA; CT, cell total SA. The results were repeated in at least three independent experiments. Data is a representative from one experiment with three samples (n=3).
3.3.4 Western blot analysis of selected α-2,6 sialyltransferase level

The LC-MS/MS results of increased cellular SAs may be in accordance with the increase of α-2,6 linked SAs on the cell surface after atorvastatin treatment as observed by flow cytometry and confocal microscopy above. Therefore, we further performed western blot analysis of the cellular α-2,6 sialyltransferases in normal and statin conditions. These sialyltransferases are grouped into two families according to the carbohydrate linkages they synthesize, including ST6Gal I and II, and ST6GalNAc I-VI [32]. ST6Gal-I for N-glycan, ST6GalNAc-II for O-glycan, and ST6GalNAc-VI for glycolipids have been investigated on immune cells [32-34]. As shown in Figure 19, ST6GalNAc-II was found highly increased, ST6GalNAc-VI no apparent change, while ST6Gal-I decreased upon atorvastatin treatment (with 20 μM for 24 h). Therefore, the elevation of ST6GalNAc-II may contribute to the increased cellular SAs and α-2,6 linked SAs on the cell surface after atorvastatin treatment. There may be other sialyltransferases related to the increase of α-2,6 linked SAs as well, which deserve a further study in future.
Figure 19: Western blot analysis of Raw 264.7 cells sialyltransferases upon atorvastatin treatment. Raw cells were incubated with 20 μM atorvastatin (or vehicle) for 24 h, and the ST6Gal-I, ST6GalNAc-II and ST6GalNAc-VI were determined by western blot analysis using rabbit anti-ST6Gal-I antibody, rabbit anti-ST6GalNAc-II antibody, and rabbit anti-ST6GalNAc-VI antibody, respectively.
3.4 Discussion

Macrophages play very important roles in the immune system related to both physiological and pathological pathways. SAs terminate most glycans on the cell surface and are involved in variety of physiological and pathological activities of immune cells. In this study, we used Raw 264.7 macrophages to comprehensively profile the sialylation status in the cell culture system including cell surface SAs levels and their linkages, secreted free, conjugated and total SAs in the culture medium and total SAs in the cells as well. First, cell surface SAs were determined by confocal microscopy and flow cytometry by using SNA and MAA labeling. As a result, α-2,3 linked SAs were found to be predominant on the Raw 264.7 macrophage surfaces, while α-2,6 linked SAs were less observed under the normal culture condition (Figure 12A). After cells were treated with atorvastatin for 24 h, α-2,3 linked SAs were unchanged, however, α-2,6 linked SAs increased significantly on cell surface (Figure 12B, Figure 13B). The wide distribution of α-2,3 linked SAs may be associated with the cell-cell interaction and signaling transduction under the normal condition. For example, sialoadhesin (siglec-1), a SA binding receptor uniquely expressed by macrophage subsets, selectively binds to only α-2,3 linked SAs [12]. While α-2,6 linked SAs may be associated with the cell-cell interaction and signaling transduction in response to external stimuli. The expression level of SAs on the cell surface is affected by its biosynthetic pathway. In particular, the synthetic enzyme, sialyltransferase, functions for adding SAs to the termini of N-linked or O-linked glycans on the cell surfaces [35]. In our study, western blot showed dramatically change of ST6GalNAc-II which may be the major contributor for the increase of cell surface α-2,6 linked SAs.
It is known that glycosylation pattern, including sialylation, changes when cells undergo apoptosis [16, 19, 20, 36-38]. The extensive studies related to SAs expression level and cell apoptosis have been done by Dr. Susan L. Bellis research group focusing on the ST6Gal-I sialyltransferase [20, 39-41]. The decrease of SA level regulated by ST6Gal-I on TNFR1 Death Receptor has been reported to induce macrophages apoptosis [20]. ST6Gal-I regulated sialylation of β1 integrins can block cell adhesion to galectin-3 and protect cells against galectin-3 induced apoptosis [40]. Moreover, Fas mediated apoptosis in colon carcinoma cells can be diminished by sialylation of the Fas death receptor by ST6Gal-I [39]. One explanation of ST6Gal-I protecting cell against apoptosis can be explained by its inhibition of galectins blinding to underling galactose, which is reported to limit the life span of variety of cells [41]. In our study, the ST6Gal-I was found to decrease significantly after cell treated with atorvastatin, and meantime the cell apoptosis level elevated dramatically. Based on Dr. Bellis report, the reduction of ST6Gal-I level can partially contribute to atorvastatin induced macrophage apoptosis. Conversely, an increase of α-2,6 linked SAs was observed in the late apoptotic lymphocytes [19]. One explanation for this phenomenon was the redistribution of immature (intracellular) membranes onto the cell surface during cell apoptosis [19]. This scenario is supported by an earlier study, which demonstrated the exposure of ER resident proteins and sugars predominantly on the incompletely processed proteins in ER and Glgi [42]. In our study, we found that α-2,6 linked SAs increased apparently on macrophage cell surface upon atorvastatin activation. The late apoptotic redistribution of the intracellular membrane may, at least in part, explain the increase of α-2,6 linked SAs on the cell surface upon atorvastatin stimulation. A further study to reveal the particular molecular mechanism is highly warranted.
Most studies on the sialylation status have been limited to SAs on the cell surface with the methods such as lectins labeling, metabolic labeling, and chemical modification [43]. Free SAs and conjugated SAs levels in the cell culture medium may change upon cell activation, such as releasing SAs from cells (surface). In addition, SAs biosynthesis may increase inside of the cells as well. Therefore, we quantitatively examined the free SAs and conjugated SAs level in the cell culture medium after atorvastatin treatment, which indicated SAs releasing fact related to cell function alteration. In addition, we quantified the total SAs in the cells as well.

So far, few studies tried to quantify the SAs trimmed off from cell surfaces [6, 44]. However, the quantification methods used were often with complicated sample preparation procedures, such as enzymatic or acid digestion, sample concentration, and SA derivatization. The DMB derivatization was mainly used for the quantification of SAs [44-48]. However, the derivatization involves both high temperature and acidic conditions, which can hydrolyze part of the conjugated SAs, and severely interrupt the quantitative accuracy of a small amount of free SAs in the cell culture medium. In our study, we developed a new LC-MS/MS method for direct quantification of SAs in the cell culture system. In this method, the free SAs in the medium can be directly analyzed after simple centrifugation, and no any sample purification or concentration step is needed. Conjugated SAs in the medium or cell lysate were efficiently hydrolyzed with 2 M acetic acid at 80 ℃ for 90 min (data is not shown) followed by direct LC-MS/MS analysis. As a result, there were no significant changes for free SAs and conjugated SAs in the medium after atorvastatin treatment, indicating that no apparent sialidases activity changed during this process. However, the amount of total cellular SAs increased from $369 \pm 29$ ng/mL to 1080
± 50 ng/mL upon cell activation. Both confocal microscope and flow cytometry studies above revealed increasing α-2,6 linked SAs on the cells, indicating that the α-2,6 linked SAs was the major contributor for the total cellular SAs increase. Our western blot analysis of sialyltrasferases showed high level of ST6GalNAc-II, indicating it may be the major contributor for the increase of α-2,6 linked SAs. Overall, the newly developed LC-MS/MS method was successfully applied to sensitively quantify both free and total SAs in the culture medium and cell lysate.
3.5 Conclusion

We profiled SAs dynamics in the whole culture system in the normal and atorvastatin treated conditions. α-2,3 Linked SAs were found predominant on the cell surface of Raw 264.7 cells under the normal condition and unchanged upon atorvastatin treated. In comparison, α-2,6 linked SAs were obscure but increased dramatically on the cell surface after atorvastatin treatment. In addition, the total SAs of the cells were significantly increased, while the SAs in the cell culture medium were unchanged after cell treated with atorvastatin. This is the first time of comprehensively analysis of cell surface SAs of their levels and linkages, free, conjugated and total SAs in the culture medium, and total SAs in the cells by combination of confocal microscopy, flow cytometry, and LC-MS/MS, which provide a sensitive, specific and systematic way to globally study SAs in the cell culture system. This work will contribute to a better understanding of the physiological and pathological roles of SAs in macrophages and the immune system as well. In general, the reported methods can be expanded to study SAs status of any kind of cells of interests.
3.6 Reference


CHAPTER IV
SIONYLATION AND DESIALYLATION DYNAMICS OF
MONOCYTES UPON DIFFERENTIATION AND POLARIZATION
TO MACROPHAGES

4.1 Introduction

Macrophages display remarkable plasticity that allows them to acquire functionally
distinct phenotypes and take part in a large number of physiological and pathological
processes [1]. Monocytes are precursors for tissue macrophages, in replenishing resident
macrophages under normal states and in response to inflammation signals by quickly
moving to the sites of infection and dividing/differentiating to macrophages in the tissues.
Further, macrophages can be phenotypically polarized to classically (M1) or alternatively
(M2) activated cells, both of them represent phenotypic extremes of macrophage
differentiation [2,3]. These cells are often characterized by the expression of their specific
cell surface protein markers, secreted cytokines and
chemokines, and transcription and epigenetic pathway. However, the precisely molecular determinants in mediating macrophage phenotypic heterogeneity and functional plasticity are still largely unknown.

A density layer of glycans is expressed on the macrophage cell surface. Lines of evidence have hinted to potential roles of cell-surface glycans in the functions of macrophages [4]. SAs are a family of 9-carbon containing acidic monosaccharides and often terminate cell surface glycans of either glycoproteins or glycolipids. The C-5-amino derivative represents the well-known neuraminic acid, and its amino functional group can be either acetylated (N-acetylneuraminic acid, Neu5Ac) or glycolylated (N-glycolylneuraminic acid, Neu5Gc). The most abundant SA is Neu5Ac. They exist on either N- or O-linked glycans, being attached to either Gal, or GalNAc units via α-2,3 or α-2,6 linkage, which are synthesized by specific enzymes [5]. Accumulated evidences indicated that cell surface SAs can dramatically impact cell properties and represent different cellular statuses [6]. Therefore, the expression levels and linkages of SAs, named as sialylation status, may be biomarkers of specific cell status/population of macrophages and may be directly involved in macrophages functions as well.

The linkage and expression level of cell surface SA are controlled by both the sialylation pathway catalyzed by specific sialyltransferse enzymes and the desialylation process catalyzed by specific sialidase enzymes and environment cues. In this study, we systematically investigated the sialylation and desialylation profiles of human monocytic leukemia cell line THP-1 after differentiation to M0 macrophages and polarization to M1, and M2 macrophages (Figure 20). THP-1 monocytes were induced with phorbol 12-myristate 13-acetate (PMA) to differentiate into macrophages (M0), and further polarized
with LPS and IFN-γ to generate M1 macrophages, with IL4 and IL13 to generate M2 macrophages [7,8]. Cell surface SA reflecting both sialyltrasferase and sialidase activities was examined by flow cytometry with specific lectins, MAA and SNA. Further, the siaoglycoconjugates synthesized inside the cells were selectively labeled with the FITC-labeled lectins and imaged by confocal microscopy. Moreover, the expression level of sialyltransferases related to the sialylation status of the cells was confirmed by western blot, and the SA amount inside the cell was determined by LC-MS/MS. On the other hand, the desialylation level of the cells was examined by measuring free SA released in the media and the sialidase activity with the exogenous substrates. Overall, profiling macrophage cell surface sialylation and desialylation status will contribute to delineate macrophage diversity and define their phenotypes and functions.
Figure 20: Systematically investigating the sialylation and desialylation profiles of human monocytic leukemia cell line THP-1 after differentiation to M0 macrophages and polarization to M1 and M2 macrophages.
4.2 Materials and methods

4.2.1 Materials

N-Acetyl-D-neuraminic acid (SA, 98%) was supplied by Rose Scientific Ltd. (Edmonton AB, Canada). N-Acetyl-D-neuraminic acid-1,2,3-\(^{13}\)C\(_3\) (\(^{13}\)C\(_3\)-SA, 99%), phorbol 12-myristate 13-acetate (PMA), lipopolysaccharides from \textit{Escherichia coli} (LPS), 4',6-diamidino-2-phenylindole (DAPI), propidium iodide (PI), RIPA buffer, protease inhibitor cocktail, rabbit anti-ST6Gal-I antibody, rabbit anti-ST6GalNAc-II antibody, rabbit anti-ST6GalNAc-VI antibody, rabbit anti-ST3Gal-IV antibody, rabbit anti-ST3Gal-VI antibody, and goat anti-rabbit IgG-peroxidase antibody were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Rabbit anti-ST3Gal-I antibody and rabbit anti-ST3Gal-V antibody were from Fisher Scientific (Hanover park, IL, USA). Rabbit anti-Human recombinant IFN-\(\gamma\), IL-4 and IL-13, and FITC labeled mouse anti-human CD11b antibody were obtained from Life Technologies (Grand Island, NY, USA). FITC labeled MAA lectin was from bioWORLD (Dublin, OH, USA). FITC labeled SNA lectin was provided by Vector Laboratories (Burlingame, CA, USA). 4-Methylumbelliferyl N-acetyl-\(\alpha\)-D-neuraminic acid (4-MU-NANA) sodium salt was purchased from Carbosynth LLC (San Diego, CA, USA). GM3 ganglioside was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Cytokine analysis was performed by EVE technologies (Calgary, Alberta, Canada).

4.2.2 Cell differentiation and polarization

THP-1 monocytes, obtained from American Type Culture Collection, were cultured in RPMI-1640 medium (Cleveland Clinic Core Facility, Cleveland, OH, USA)
supplemented with 10% fetal bovine serum (Life Technologies, Grand Island, NY, USA) and 1% penicillin/streptomycin (Cleveland Clinic Core Facility, Cleveland, OH, USA) in a humidified atmosphere containing 5% CO2 at 37 °C. The differentiation of THP-1 monocytes to macrophages was accomplished by PMA stimulation for 48h. To generate M1-polarized macrophages, LPS (100 ng/mL) and IFN-γ (20 ng/mL) were added at the last 18 h of PMA treatment. To generate M2-polarized macrophages, IL4 (20 ng/mL) and IL13 (20 ng/mL) were added at the last 18 h of PMA treatment [7, 8]. In order to quantify SA in the medium, FBS was removed from culture medium before the addition of PMA.

4.2.3 Flow cytometric analysis

To determine cell surface SAs, cells were washed with cold PBS, and then incubated with 50 μL of FITC labeled MAA (10 μg/mL) or SNA (5μg/mL) for 15 min at room temperature in dark. Cells were subsequently washed, re-suspended in cold PBS and analysed by a FACSCanto II flow cytometer operated through BD FACSDiva software (BD Bioscience, Mountain View, CA, USA). For the CD11b staining, cells were washed, suspended in cold FACS buffer (PBS, 0.5% BSA, and 0.1% sodium azide), and blocked with 10% heat inactivated human serum for 20 min on ice. Cells were then centrifuged and suspended in 100 μL cold FACS buffer with 5 μL FITC labeled CD11b antibody. After 30 min incubation on ice, cells were washed and re-suspended in cold FACS buffer for flow analysis.
4.2.4 Confocal microscopy

THP-1 monocytes were seeded on cover slips in 6-well plate at a density of one million cells/well, and then differentiated and polarized to different type of macrophages following the method described in the Cell Differentiation and Polarization section. To determine SA expression of the macrophages, cells were washed with cold PBS, and fixed with 4% PFA for 10 min. Excess PFA was removed, and optionally the cells were permeabilized with 0.2% TritonX-100 for 10 minutes to expose SAs inside the cells. Then cells were stained with MAA-FITC (10 μg/mL) or SNA-FITC (5 μg/mL) for 15 min, followed by 2 min DAPI stain. Unbound lectin and DAPI were washed off with PBS, and cover slips were mounted on ProLong Gold Antifade reagent. Images were acquired at a 60 × magnification using a Nikon A1Rsi confocal microscope, and data analysis was performed using NIS-Elements software (Nikon Instruments Inc., Melville, NY, USA). To determine SAs expressed on THP-1 monocytes, the cover slips placed in 6-well plate were first treated with 1% alcian blue solution (Electron Microscopy Sciences, Hatfield, PA, USA) for 30 min, and then one million of cells in PBS buffer were seeded in each well. After standing for 20 min, the cells deposited on cover slips were fixed with 4% PFA and then followed the steps for macrophages staining.

4.2.5 LC-MS/MS analysis

SAs in the media and cell lysates were quantified according to our established protocol [9]. Briefly, the chromatography was performed on a Nexera liquid chromatography system (Shimadzu, Columbia, MD, USA). The separation of SA and $^{13}$C$_3$-SA (internal standard, IS) was carried out by a Primesep D column (2.1 x 100mm, 5 μm;
SIELC Technologies, Prospect Heights, IL, USA) in a binary gradient mode with phase A containing 10 mM ammonium formate and 0.1% formic acid in water, and phase B composed of 90% acetonitrile, 10% water, 3 mM ammonium formate, and 0.04% formic acid. The gradient elution started at 10% phase B and changed linearly to 100% B from 1.1 min to 4.0 min. To equilibrate the column, the gradient sharply dropped to 10% B from 6.0 min to 6.1 min and maintained for 3 min. The MS detection was carried out by a Qtrap 5500 mass spectrometer equipped with electrospray ionization source (AB SCIEX, Framingham, MA, USA). The quantitative transitions were set at m/z 308 → 87 for SA, and 311 → 90 for IS. The data acquisition and processing were conducted using Analysis 1.6.1 software. The calibration range for SA was from 2.00 to 1.00×10³ ng/mL.

To quantify free SA in the medium, 95 μL of culture medium was spiked with 5 μL of IS, and then 5 μL of the mixture was subjected to LC-MS/MS analysis. The total SA in culture medium was determined by hydrolyzing the sample with 2 M acetic acid (1:1) at 80 °C for 90 min, and then mixing 95 μL of the lysate with 5 μL of the IS before analysis. For quantification of cell lysate SA, the sample at a density of 1×10⁶ cells/mL was untrosonicated to obtain homogeneous mixture and then followed the procedure of total SA quantification for culture medium.

4.2.6 Sialidase activity measurement

Cells in the indicated conditions were collected and two millions of the cells were suspended in the reaction buffer, which containing 0.05 M sodium acetate at pH 4.4, 0.1% Triton X-100, and 0.1% BSA. The activity of NEU1 and NEU3 were determined by the addition of 0.125 mM 4-MU-NANA and 0.250 mM ganglioside GM3, respectively.
12]. After 1 h incubation at 37 °C, the reaction mixtures were centrifuged to remove cellular debris, and the supernatants were precipitated with acetonitrile at 1:3 ratio to remove the soluble proteins. Then 20 μL of the supernatant was mixed with 5 μL of IS and 75 μL of PBS, and 5 μL of the mixture was subjected to the LC-MS/MS analysis. Protein concentrations in each assay were measured by the Bradford method using a protein assay kit (Bio-Rad, Hercules, CA, USA). One unit of sialidase activity was defined as one nmol of free SA that released from one mg of total protein per hour at 37 °C.

### 4.2.7 Western blot

Cells in the indicated conditions were lysed with a mixture of RIPA buffer and protease inhibitor cocktail (1:100, v/v) on ice for 5 min, and the cell lysates were collected and clarified by centrifugation at 15,000 g at 4 °C for 10 min. Protein concentrations were measured by Bradford method using a protein assay kit. Proteins at amount of 30 μg/sample were separated on SDS-12% polyacrylamide gels and then transferred to polyvinylidene difluoride membrane. The membranes were blocked with 5% nonfat milk for 1 h at room temperature, followed by incubation with primary antibodies at 4°C overnight. After washing with tri-buffered saline containing 0.05% Tween 20 (TBS-T) buffer, the membrane was probed with specific secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature. Protein detection was accomplished by using the ECL Prime kit (Pierce) with the LI-COR Odyssey Fc imaging system.
4.3 Results

4.3.1 Cell surface SA analyzed by flow cytometry

To determine the cell surface sialylation level during THP-1 monocytes differentiation, cells were incubated with PMA at 10, 20, and 50 ng/mL for 48 h. The differentiation of THP-1 monocytes to macrophages was confirmed by the expression of CD11b, which showed more than 80% positive after 48 h treatment with PMA (data is not shown) [13, 14]. SA on the cell surface is found most commonly linked to underlying sugars with α-2,3 and α-2,6 linkages [15]. To examine the cell surface sialylation status, we performed flow cytometry to distinctively measure SA with different linkages on the THP-1 monocytes and macrophages by using MAA-FITC and SNA-FITC labeling, which selectively recognize α-2,3 and α-2,6 linked SA, respectively. As observed from Figure 21 A and B, both MAA and SNA peaks left shifted after cells treated with PMA, suggesting the decrease of both α-2,3 and α-2,6 linked SA on the cell surface of the differentiated macrophages. The statistical analysis showed the mean fluorescent intensity (MFI) of MAA labeled α-2,3 SA decreased more than 20% and SNA labeled α-2,6 SA decreased more than 30% (Figure 21 C). The change of cell surface SA was not related to PMA concentration as observed in Figure 21 C.
Figure 21: Cell surface SAs determined by flow cytometry. (A): THP-1 monocytes were labeled with MAA-FITC (10 μg/mL) or SNA-FITC (5 μg/mL). (B): M0 macrophages were obtained by differentiation of THP-1 monocytes with PMA at 10 ng/mL for 48 h, and then the cells were labeled with MAA-FITC (10 μg/mL) or SNA-FITC (5 μg/mL). (C): Statistical analysis of three independent experiments (n=3). Significantly different values (*P < 0.05, **P < 0.01) were obtained by SPSS 19.0 with paired-samples t test.
Next, the polarization of M0 macrophages was carried out by the stimulation with M1 cytokines (LPS and IFN-γ) or M2 cytokines (IL-4 and IL-13) with PMA at concentrations of 10, 20, and 50 ng/mL, respectively. Cytokines including GM-CSF, IL-1β, IL-6, IL-12, and TNFα were analyzed before and after polarization (Figure 22). The change of cell surface SA was examined by flow cytometry by using MAA-FITC and SNA-FITC labeling too. As a result, no significant peak shift was observed for either MAA or SNA labeling compared to M0 macrophages (data is not shown), suggesting the cell surface SA content was unchanged, which may be not crucial during the macrophage activation. The sialidase from *Arthrobacter ureafaciens* was used to release cell surface SA from M0, M1 and M2 macrophages. However, due to the insufficient releasing of α2,6-linked SA in the neutral pH, no further study was performed with exogenous sialidase (Figure 23).
Figure 22: Cytokine analysis of THP-1 derived M0, M1 and M2 macrophages (n=3). THP-1 monocytes were seeded in 12-well plate at a density of 0.5 million cells per well. After differentiation with PMA at 10 ng/ml and polarization with M1 and M2 cytokines, GM-CSF, IL-1β, IL-6, IL-12, and TNFα in the medium were quantified with human cytokine array offered by EVE technologies. As a result, M1 has higher concentrations in IL-1β, IL-6, IL-12, and TNFα compared to M0 and M2. While M0 and M2 show similar cytokine profile, except granulocyte-macrophage colony-stimulating factor (GM-CSF), which is a specific marker for M1.
Figure 23:  Cell surface SA profile after sialidase treatment. THP-1 derived M0, M1 and M2 macrophages were incubated with sialidase from *Arthrobacter ureafaciens* in PBS buffer for 1h. Then α2,3-linked SA was labeled with MAA-FITC (10 μg/mL), and α2,6-linked SA was labeled with SNA-FITC (5 μg/mL). The nuclei were stained with DAPI (1 μg/mL). As is observed, α2,3-linked SA is more prone to sialidase treatment, while α2,6-linked SA shows a less releasing efficiency.
4.3.2 Cellular SA expression determined by confocal microscopy

To visualize the SA expression, the cells were permeabilized to detect SA inside the cells and without permeabilization to determine the cell surface SA only. As shown in Figure 24 B and D, both MAA labeled α 2,3-linked SA and SNA labeled α2,6-linked SA were found highly expressed inside the cells after PMA treatment for 48 h, indicating that the sialylation became more active in the differentiated macrophages, and this change was not related to PMA concentrations (data is not shown). However, all the M0, M1, and M2 macrophages showed a similar expression level of SA inside the cells. We were not able to determine any difference in fluorescent intensity of cell surface SA between monocytes and M0, M1, and M2 macrophages (Figure 24 A and C).
Figure 24: The SA expression level examined by confocal microscopy. The differentiation of THP-1 monocytes was carried out by incubation with 10 ng/mL of PMA for 48 h. M1 and M2 polarizing cytokines were added at the last 18 h of PMA differentiation. Cells in (B) and (D) groups were permeabilized with Triton X-100 prior to MAA-FITC (10 μg/mL) or SNA-FITC (5 μg/mL) stain. (A): α2,3-linked SA on the cell surfaces was labeled with MAA-FITC. (B): Cells were permeabilized first, and then α2,3-linked SA on the cell surfaces was labeled with MAA-FITC.
linked SA on the cell surfaces and inside the cells was labeled with MAA-FITC. (C): α2,6-linked SA on the cell surfaces was labeled with SNA-FITC. (D): Cells were permeablized first, and then α2,6-linked SA on the cell surfaces and inside the cells was labeled with SNA-FITC. The results were reproduced at least in three independent experiments. The scale bar represents 10 μm.
4.3.3 LC-MS/MS quantification of SA in the culture media and cell lysates

The SA concentrations in the culture media and cell lysates were measured by LC-MS/MS. The results are listed in Figure 25, which shows the dramatic increase of SA in the cell culture media and cell lysates with PMA treatment in 48 h. For example, after cells incubated with 10 ng/mL PMA for 48 h, the free SA in the medium reached to 12.83 ± 0.70 ng/mL compared with 4.18 ± 0.05 ng/mL in non-differentiated cells; the total SA in the medium increased in a similar manner, which changed from 28.13 ± 1.36 ng/mL to 73.50 ± 2.55 ng/mL. The SA in the cell lysate at a density of 1 × 10^6 cells/mL was found to be 728 ± 17 ng/mL in the monocytes and increased to 1310 ± 203 ng/mL in the differentiated macrophages. However, the concentration of PMA did not show any significant effect on the SA concentration in either culture media or cell lysates.

Upon polarization of M0 macrophages with M1 and M2 cytokines, no significant change of SA in the medium was found (Figure 25 D and E). While SA in the cell lysates changed depending on the initial PMA concentration used to differentiate monocytes. The SA concentration in M1 macrophages decreased significantly compared with M0 macrophages at the PMA concentration of 50 ng/mL while the SA amount increased in the M2 macrophages, and the significant difference was found at PMA concentrations of 20 and 50 ng/mL.
Figure 25: SA levels in the cell culture media and cell lysates determined by LC-MS/MS. Free SA in the media was determined by LC-MS/MS directly. The medium samples were hydrolyzed with acetic acid prior to LC-MS/MS analysis to quantify the total...
SA. For cell lysate SA quantification, cells were ultrasonicated first to obtain homogeneous mixture, and then followed by acid hydrolysis and LC-MS/MS analysis. (A): Free SA in the PMA differentiated macrophages (M0) culture media. Cells were cultured at a density of 1 million cells/2 ml medium and treated with vehicle (concentration 0 ng/mL) or PMA at concentrations of 5, 10, 20, 50, 100 ng/mL. The media were collected at 24, 48, and 72h for LC-MS/MS analysis. (B): Total SA in the M0 macrophages culture media. (C): The SA in the M0 cell lysates. (D): Free SA in the M0, M1 and M2 macrophages culture media. Cells were incubated with PMA at concentrations of 10, 20, and 50 ng/mL for 48 h. The M1 and M2 polarizing cytokines were added in the last 18 h of PMA differentiation. (E): Total SA in the M0, M1 and M2 macrophages culture media. (F): The SA in the M0, M1, and M2 cell lysates. The results were repeated in at least three independent experiments. Data is a representative from one experiment with three samples (n=3). The statistical analysis was performed using SPSS 19.0 with paired-samples t test. *P < 0.05, **P < 0.01.
4.3.4 Sialidases activity assay

During the differentiation of THP-1 monocytes to macrophages, free SA in the culture medium increased in all three conditions after 48 h treatment, which was corresponded with the decease of SA on the cell surface. The decreasing could be attributed to the elevated desialylation process, which was catalyzed by specific sialidases located in lysosomal, cytosol and cell surface. It was known that the lysosomal Neu1 and plasma membrane-associated Neu3 are two major forms of sialidases in mammalian cells [16]. In this study, the sialidase activity of Neu1 and Neu3 in THP-1 monocytes and M0 macrophages was determined using the exogenous sialidase substrates 4-methylumbelliferyl N-acetyl-α-D-neuraminic acid (4-MU-NANA) sodium salt and ganglioside GM3, respectively [10-12, 17]. For control analysis, the cells were incubated with only reaction buffer to determine the desialylation level of endogenous sialylconjugates under the same condition. As shown in Figure 26, both endogenous group and 4-MU-NANA group indicate the sialidase activity increased significantly after 24 h differentiation, and the highest activity reached in 48 h. For example, after 48 h differentiation the sialidase activity increased from 0.97 ± 0.02 units to 5.08 ± 0.03 units in endogenous group, from 12.01 ± 0.54 units to 28.48 ± 2.28 units in 4-MU-NANA group, while from 4.17 ± 0.29 units to 5.97 ± 0.38 units in the GM3 group. These data suggest Neu1 is the major contributor for the sialidase activity during THP-1 monocytes differentiation. Since no apparent change of free SA in the medium during M1 and M2 macrophages polarization, and thus the sialidase activity was not examined in these processes.
Figure 26: Comparison of sialidase activity in THP-1 monocytes and PMA differentiated M0 macrophages. The monocytes were differentiated with PMA at 10 ng/mL, and the sialidase activity was measured at 24, 48, and 72 h with 4-MU-NANA and GM3 as substrates for NEU1 and NEU3, respectively. In the endogenous group, cells were incubated with only reaction buffer, and the results represented sialidase activity with endogenous sialylconjugates as substrates. This results were repeated in three independent experiments. Data is a representative from one experiment with three samples (n=3). Significantly different values (*P < 0.05, **P < 0.01) were obtained by SPSS 19.0 with paired-samples t test.
4.3.5 Sialyltransferase expression level determined by Western blot

The LC-MS/MS results of increased cellular SA were in accordance with the enhanced sialylation inside the cells as observed by confocal microscopy above. Therefore, we performed Western Blot assays to distinctively study the effect of sialyltransferases in THP-1 monocytes and the differentiated and polarized macrophages. Sialyltransferases for α2,3-linkages, including ST3Gal-I for O-glycan, ST3Gal-IV for both O-glycan and N-glycan, ST3Gal-V for glycolipid, and ST3Gal-VI for N-glycan, were compared. For α2,6-linked SA synthesis, sialyltransferases including ST6Gal-I for N-glycan, ST6GalNAc-II for O-glycan, and ST6GalNAc-VI for glycolipids were investigated. As a result (Figure 27), both ST3Gal-I and ST3Gal-V showed a low expression level in the monocytes, but increased in the M0, M1 and M2 macrophages. While ST3Gal-IV and ST3Gal-VI were not detected in all the cells, which may indicate the low abundance of α2,3-sialylated N-glycans in THP-1 cells. In terms of α2,6-linked SA, ST6Gal-I and ST6GalNAc-II increased the expression in the macrophages compared to the undifferentiated monocytes. However, the expression of ST6GalNAc-VI decreased dramatically in the macrophages. The tremendous change of ST3Gal-V and ST6GalNAc-VI suggests the vast reorganization of sialylated glycolipids during monocytes differentiation and polarization.
Figure 27: Western blot analysis of sialyltransferases in THP-1 monocytes and the derived macrophages. THP-1 monocytes were incubated with 10 ng/mL of PMA for 48 h to obtain M0 macrophages. The M1 and M2 macrophages were polarized by addition of the corresponding cytokines in the last 18 h of PMA differentiation. Sialyltransferases including ST3Gal-I, ST3Gal-V, ST6Gal-I, ST6GalNAc-II, and ST6GalNAc-VI were determined by western blot using rabbit anti-ST3Gal-I antibody, rabbit anti-ST3Gal-V antibody, rabbit anti-ST6Gal-I antibody, rabbit anti-ST6GalNAc-II antibody and rabbit anti-ST6GalNAc-VI antibody, respectively.
4.4 Discussion

Monocytes originate in the bone marrow and are then released into the peripheral blood, where they circulate for several days before differentiation to macrophages, contributing to the host defense and tissue remodeling and repair [18]. SA terminates most of the glycan structures on the cell surfaces and secreted glycoconjugates. Consequently, it is involved in variety of physiological and pathological activities of immune cells. However, so far there is no systematic study related to the sialylation and desialylation status of monocytes and the corresponding macrophages. In this study, THP-1 monocytes were used to comprehensively profile the SA level change during monocytes differentiate to M0 macrophages and further polarize to M1 and M2 macrophages. The cell surface SA in both $\alpha$2,3- and $\alpha$2,6-linkages were decreased during the process, and this result was further supported by the elevation of cellular Neu1 activity and increase of free SA concentration in the medium. Next, SA synthesis increased in the macrophages, which was indicated by confocal images of lectin labeled siaoglycoconjugates inside the cells and the SA amount inside the cells determined by LC-MS/MS. These findings were further supported by the change of sialyltransferases with Western Blot assays which showed the elevated expression of ST3Gal-I, ST3Gal-V, ST6Gal-I, and ST6GalNAc-II while the down regulation of ST6GalNAc-VI (Figure 27). It was found that Monocyte-characteristic surface sialoglycoproteins disappear during in vitro differentiation of monocytes to adherent macrophages, which was considered to a parameter for the conversion of monocytes to macrophages [19]. Our flow cytometry results were consistent with this finding, that showed the decrease of both $\alpha$2,3- and $\alpha$2,6-linked SA on the cell surfaces during THP-1 monocytes differentiate to macrophages.
A previous study focused on the endogenous sialidases of human monocytes during differentiation to macrophages show a significant increase of NEU1 and NEU3 as analyzed by PCR and Western Blot [12]. In our study, we provide the first evidence that the increase of Neu1 activity is associated with the increase of free SA released in the cell culture medium. Neu1 catalyzes lysosomal catabolism of sialylated glycoconjugates. During the differentiation of monocytes or the monocytic cell lines, THP-1, to macrophages, the majority of Neu1 were translocalized from lysosomes to the cell surface. The suppression of Neu1 expression in the THP-1 derived macrophages by small interfering RNA or with anti-Neu1 antibodies was reported significantly reduced the ability of the cells to engulf bacteria or to produce cytokines [20]. Whereas, treatment of cells with purified Neu1 was able to reduce surface sialylation and restore the phagocytic ability [21]. However, there is no difference between medium free SA of M0, M1, and M2 macrophages. This observation suggests the similarity of sialidase activity between these subtypes of macrophages.

The relative activity of sialyltransferases influences the expression of sialylated molecules on the cell surfaces and secreted sialoglycoconjugates, and may contribute to the definition of the glycosylation pattern of monocytes and the corresponding macrophages. However, no systemic studies have focused on the SA pattern changing along monocytes differentiation and polarization so far. In our study, the increasing of the cellular SA level in the differentiated M0 macrophages was confirmed by both LC-MS/MS and confocal microscopy, while the difference between M0, M1, and M2 macrophages could be only distinguished by LC-MS/MS on the cellular SA level, which is possibly due to the sensitivity of the different detection method. The Western Blot results showed sialyltransferases, including ST6Gal-I, ST6GalNAc-II, ST6GalNAc-VI, ST3Gal-I, and
ST3Gal-V, contributed to the SA variation in the differentiated and polarized macrophages. The elevation of ST6Gal-I and ST3Gal-I indicated the enhanced synthesis of α2,6-sialylated N-glycans and α2,3-sialylated O-glycans, respectively. Similar to our observation, both enzymes were found upregulated during monocytes differentiate to dendritic cells (DCs) at their mRNA level [22]. ST6GalNAc-II has been reported to be increased in the LPS treated human macrophages and atorvastatin treated Raw macrophages, suggesting the increase expression of α2,6-sialylated O-glycans may be related to cell activation status [9, 23]. ST3Gal-V is responsible for the formation of GM3 using lactosylceramide as the substrate, and previous reports showed the enhancing of GM3 expression during differentiation of monocytes to macrophages [24, 25], which is in accordance with the rise of ST3Gal-V in our findings. ST6GalNAc-VI catalyzes the formation of α2,6-sialyltransferase involved in the synthesis of all α-series gangliosides, and the decrease of this enzyme level may be related to the well-recognized capability of macrophages to respond and alter a wide range of lipid products [26]. In our study, both ST3Gal-IV and ST3Gal-VI that catalyzing the formation of α2,3-linked SA on N-glycans were undetectable although the mRNA expression was reported in human peripheral blood monocytes [22]. This observation may result from either the low abundance of α2,3-linked SA on N-glycans, or the low sensitivity of the corresponding antibodies on the market.

M1 macrophages showed a decreased level of cellular SA, whereas M2 macrophages showed increased in compared to M0 macrophages. This interesting phenomenon may indicate the cell function change during cell differentiation. M1 macrophages are known to be associated with acute inflammatory responses and M2 macrophages are characterized with inflammatory responses and adaptive type I immunity.
Previous studies indicated that the decrease of sialylation level is always associated with the increase of inflammatory response and vice versa [20, 21, 30-33]. For example, the anti-inflammatory activity of immunoglobulin G (IgG) is resulted from the sialylation of its Fc core polysaccharide, which could be reduced upon the induction of an antigen-specific immune response. Thus the differential sialylation may provide a switch from innate anti-inflammatory activity in the steady state to generate adaptive pro-inflammatory effects upon antigenic challenge [30]. In term of immune cells, the suppression of Neu1 expression in THP-1 derived macrophages can significantly reduce the ability of the cells to engulf bacteria or to produce cytokines, while this inflammatory function can be restored by the treatment of sialidase [20, 21].
4.5 Conclusion

We report a systematic investigation of sialylation and desialylation dynamics of monocytes after differentiation and polarization to macrophages. The lectin labeling, followed with flow cytometry and confocal microscopy detection gives the direct evidence of SA change regarding to α-2,3 and α-2,6 linkage. Moreover, LC-MS/MS provides a sensitive and accurate detection of released SAs in both culture medium and SAs synthesized inside the cells. Further, studies based on the levels of sialidases and sialyltransferases furnish a fundamental mechanism leading to the alteration of SA, which could be used to manipulate the differentiation and polarization of monocytes. This work will contribute to a better understanding of the physiological and pathological roles of SAs in the monocyte differentiation and polarization as well as the immune system.
4.6 References


CHAPTER V
SUMMARY

SAs often exist as the terminal sugars of glycan structures of cell surface glycoproteins and glycolipids. The level and linkages of cell surface SAs, which are controlled by both sialylation and desialylation processes and environment cues, can dramatically impact cell functions and represent different cellular statuses. Traditional approach for SAs study employed the SA binding proteins, lectins, which can specifically recognize SAs in certain linkages. However, the application of lectins is almost limited to the intact cells, and the bias in differentiation the change of SAs on the cell surface cannot be avoid due to the fact that SAs may exist both inside and outside the cell membrane, and also the spacial hindrance cannot be avoided. SA quantification was used in some researches to study SA amount change in different conditions. Quinoxilinone derivatization provides a sensitive and specific way to study total SA, as well as SA species. However, the high temperature and acidic condition make it impossible to analyze only
the free SA part in matrix, such as cell culture medium and plasma, which contains large ratio of conjugated SA. Although several direct quantification methods had been developed for free SA determination, the most sensitive one reported had quantification limit of 100 ng/mL and most of others could only detect SA in μg/ml. These methods may be good for patient samples with SSDs, which accumulated large amount of free SA, but not applicable for regular cell culture medium generally with free SA less than 10 ng/mL.

In my study, firstly I developed two new LC-MS/MS methods for broad scope of SA quantification. The derivatization approach using a stable and inexpensive reagent DAT provides a stable product of SA with high MS response, proving a convenient and cost-effective LC-MS/MS analysis of conjugated SA and free SA after protein depletion. In addition, trace amount of SA in the reagents including water was found in this study, which made it inaccurate to quantify low concentration of SA. Therefore, the $^{13}$C$_3$-SA was used as calibration standard to ensure the accuracy of the measurement. Last, this assay used UHPLC for separation of native/labeled SA and IS from matrix interference, and employed mass spectrometry in MRM mode for sensitive and selective detection. We have achieved a LLOQ of 20 ng/mL and a total running time of 4.2 min, which is the most sensitive and quick measurement for SA with derivatization. This method has been applied to quantify free SA in plasma samples from cardiovascular disease patients. We have found the increase of free SA in these patients compared to health individuals. However, more samples are required to make statistical conclusion.

In order to work on large scale of samples, we further improved SA quantification with a non-derivatization method. In this direct LC-MS/MS approach, cell culture medium can be analyzed directly after simple centrifugation, which provides the most accurate way
for free SA detection. This method is sensitive enough to quantify SA in 2 ng/mL in culture medium. However, one limitation for this method is the capability to separate SA analogues. It affords excellent resolution for Neu5Ac and Neu5Gc, while doesn’t work well when Neu5Ac loses the first carbon, which is the proposed physiological pathway for desialylation under oxidative stress; also it shows no retention when the hydroxyl group on the C-5 in Neu5Gc is substituted with glycine. However, all these problems can be resolved by DAT derivatization with LC-MS/MS quantification.

LC-MS/MS has been proved to be a powerful tool to quantitatively study SA in the cell lysate, and both free and total SA in the culture medium. With the combination of flow cytometry and confocal microscopy using fluorescent lectins labeling for the intact cells, the sialylation status on the cell surface and inside the cells can be revealed, which offers a powerful approach to comprehensively study sialylation status in different cellular conditions related to both physiological and pathological processes. With these approaches, we performed globally profiling of the sialylation status of macrophages and their release of SAs in the cell culture medium after atorvastatin treatment. Both flow cytometry and confocal microscopy results showed that α-2,3 linked SAs were predominant on the cell surface and changed slightly upon treatment with atorvastatin, while α-2,6 linked SAs were negligible in the normal culture condition but significantly increased after treatment. Meanwhile, the amount of total cellular SAs increased about three times upon treatment as determined by LC-MS/MS method, which further supported the observation from flow cytometry and confocal microscopy. These results indicated that the cell surface α-2,6 sialylation status of macrophages changes distinctly upon atorvastatin stimulation, which may reflect on the biological functions of the cells. Moreover, the increase of α-2,6 linked
SA correlates well with the Annexin V positive cell ratio, which suggests a possible correlation of SA and cell apoptosis.

Next, we examined the sialylation and desialylation features during monocytes differentiate to macrophages. In this study, THP-1 monocytes were used as model cell line to differentiate to inactivated macrophages (M0), then polarize to M1 and M2 macrophages under different stimuli. Interestingly, both α-2,3 and α-2,6 linked SAs on the cell surface were found to be decreased after monocytes were differentiated to macrophages, which was in accordance with the increased level of free SA in the cell culture medium and the elevated activity of endogenous NEU1 sialidase. Meanwhile, the siaoglycoconjugates inside the cells increased as confirmed by confocal microscopy and the total SA inside the cells increased as determined by LC-MS/MS. Western blot analysis showed higher expression levels of sialyltransferases, including ST3Gal-I, ST3Gal-V, ST6Gal-I and ST6GalNAc-II. Further, upon polarization, the cell surface sialylation levels of M1 and M2 macrophages remained the same as M0 macrophages, while a slight decrease of cellular SAs in the M1 macrophages but increase in the M2 macrophages were confirmed by LC-MS/MS.

In summary, we developed two LC-MS/MS methods for broad scope of SA quantification. The derivatization method has been successfully applied to determine free SA in human plasma. The direct quantification approach has been combined with flow cytometry and confocal microscopy with lectin labeling to profile SAs in the cell culture system. LC-MS/MS can accurately quantify SA in the cell lysate and culture medium in a high throughput fashion. The SA linkages on the cell surface can be distinguished by flow cytometry and confocal microscopy with specific lectin labelings. The total SA amount in
the cell lysate provides a direct evidence of the consequence of sialyltransferases change, and the free SA in the medium can serve as an indicator for sialidases activity.
6.1 SAs related glycomics study

Glycomic analyses seek to understand how a collection of glycans relates to a particular biological event. As described before, sialylated glycoconjugates participate in many biological processes, from intracellular signaling to organ development and tumor growth. However, due to the fact that the understanding of the molecular functions of SAs in these physiological and pathological processes is just emerging, it is not surprising that a clear understanding of the significance of nature’s SAs structural diversity is still missing. Our current studies have provided the evidence that SA amount and the linkages of the cells vary under different conditions. To understand the underlying molecular mechanism and reveal new functions of sialylated structure in cell-cell communication, it will be valuable to provide the detail sialylated glycan structure corresponding to different cellular conditions.

High-resolution mass spectrometry is the primary technique for characterizing the structures of individual glycans when only small quantities are available, as is the case of
most glycomic studies. In this typical experiment, the sialylated glycoproteins from cell lysates can be enriched by lectin affinity column. Then the N-glycans are selectively released by endoglycosidase such as PNGase F, and the O-glycans are chemically released by reductive elimination due to no endoglycosidase has been found to work on O-glycan. The released glycan can be further permethylated to increase the MS response and to protect the SA structure. Alternatively, the glycans can be analyzed directly under negative mode or under positive mode after enzymatic desialylation. The detailed glycan structures can be elucidated by the combination of fragmentation pattern and further treatment with specific glycosidases.

In my study, I have worked with bovine fetuin as a standard glycoprotein, and the released glycans were analyzed by Bruker Autoflex III MALDI-TOF-TOF mass spectrometry (Matrix Assisted Laser Desorption/Ionization-Time of Flight). As shown in Figure 28, this instrument is sensitive to detect glycoproteins on microgram scale with both native glyans or permethylated glycans. However, no glycans could be identified from the membrane proteins of THP-1 monocytes, which is likely due to the insufficient sample amount. In future, mass spectrometry with higher sensitivity could be utilized to further this study.
Figure 28: MALDI-TOF-MS spectra of glycans. (A) Permethylated $N$-glycans from 30 μg fetuin. (B) Desialylated native $N$-glycans from 30 μg fetuin. (C) A $N$-glycan sample released from membrane protein of 10 million THP-1 cells.
6.2 Systematic and site-specific analysis of sialated proteins on the cell surface of immune cells

Metabolic oligosaccharide engineering is a methodology that originated over two decades ago when the sialyltransferases were found to have the capacity to add modified SAs to glycoconjugates. This technology took a dramatic step forward when the modified ManNAc analogs were found to be able to incorporate into sialylated glycans. With this approach, the biorthogonal functional groups such as azide on the ManNAc or Neu5Ac analogs could be incorporated on the cell surface (Figure 29). Thus this functional group can further undergo covalent reaction with exogenous agents bearing complementary functionality which could enable systematic and site-specific analysis of sialated proteins on the cell surface of immune cells.

The vast remodeling of cell surface sialoglycoproteins in the immune cells under different activation conditions has been reported in many researches. With the metabolic oligosaccharide engineering approach, the systematic and site-specific analysis of sialated proteins on the cell surface can be achieved. Specifically, the unnatural SA containing bioorthogonal functional group such as azide can be incorporated on the cell surface, and the glycans bearing this special sugar can be tagged by other agents to achieve labeling and furnish later separation. Then the cells can be lysed, and the labeled glycoconjugates can be pulled out with specific beads. These purified sialoglycans can be indentified by MS after PNGase F digestion. Further, if this enzymatic step is carried out in heavy water, all the Asn is converted to Asp with an $^{18}$O tag that can be identified by MS. Overall, this combined glycomics and metabolic engineering approach in immune cells has a great
potential in identifying new targets for disease treatment and vaccine development, and discovering effective biomarkers for disease detection and surveillance.
Figure 29: The principle of the site-specific identification of the cell surface N-sialoglycoproteome by integrating metabolic labeling and MS-based glycomics techniques. (Chen W, Smeekens JM, Wu R. 2015. Systematic and site-specific analysis of N-sialoglycosylated proteins on the cell surface by integrating click chemistry and MS-based proteomics. Chem Sci. 6: 4681-4689.)